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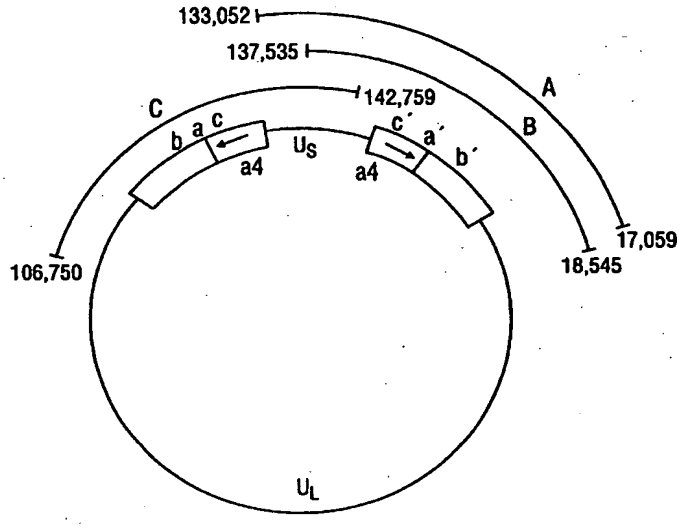
(54) Title: HERPES SIMPLEX VIRUS Us3 AND ICP4 AS INHIBITORS OF APOPTOSIS

(57) Abstract

The ICP4 protein of herpes simplex virus plays an important role in the transactivation of viral genes. The present invention discloses that ICP4 also has the ability to inhibit apoptosis. This function appears to reside in functional domain distinct from the transactivating function, as indicated by studies using temperature sensitive mutants of ICP4 that have transactivating function at elevated temperatures. Also disclosed are methods for inhibition of apoptosis using ICP4 or an ICP4 encoding gene, such as an α4 gene, methods for inhibiting ICP4's apoptosis–inhibiting function, and methods for the production of recombinant proteins and treatment of HSV infections. Further, the present invention discloses that the HSV–1 mutant lacking the α4 gene, has a secondary mutation in the gene Us3 specifying a protein kinase. Thus a functional Us3, a viral gene encoding a protein kinase known to phosphorylate serine/threonine within a specific arginine rich consensus sequence, is required in order to block apoptosis. Also disclosed are methods for inhibition of apoptosis using Us3 or an Us3 encoding gene, methods for inhibiting Us3's apoptosis–inhibiting function, and methods for the production of recombinant proteins and treatment of HSV infections.



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DESCRIPTION

HERPES SIMPLEX VIRUS U_S3 AND ICP4 AS INHIBITORS OF APOPTOSIS

BACKGROUND OF THE INVENTION

The government may own certain rights in this application by virtue of federal funding under grant numbers AI124009 (NIAID) and CA47451 (NCI).

I. Field of the Invention

The present invention relates to the fields of molecular and cell biology generally, and more specifically, it addresses mechanisms for growth control in eurkaryotic cells. In particular, there are provided viral genes that inhibit normal cell death and methods for use thereof.

II. Related Art

The control of host cell gene expression, and often the control of genes involved in DNA replication, are integral parts of the life cycle of a virus. However, recent evidence suggests that most eukaryotic cells respond to viral disruption of normal cellular physiology by undergoing programmed cell death (apoptosis) (White, 1993). To counteract this, many viruses have evolved mechanisms to block host cell death (Clem and Miller, 1994; White and Gooding, 1994). In several cases, viral genomes have been found to contain genes whose products interact with proteins that play a central role in regulating cell survival.

Programmed cell death is triggered by several factors and may take various forms. For example, the synthesis of double-stranded RNA activates kinases which phosphorylate the α subunit of eIF-2 and completely turn off protein synthesis (Sarre, 1989). Ultimately, activation of metabolic pathways causes a pattern of morphological, biochemical, and molecular changes which result in cell death without spillage of cellular constituents which would result in an inflammatory response detrimental to the host (Wyllie, *et al.*).

Apoptotic cell death is commonly observed during embryogenesis and organ involution and in the natural death of terminally differentiated cells at the end of their life span. Most viruses which induce either the shut-off of protein synthesis or apoptosis also have evolved mechanisms which block host responses and enable them to replicate in their hosts (Shen and Shenk, 1995).

Among the best-known examples of viral gene products which block apoptosis are the adenovirus E1B M_r19,000 protein (Rao, *et al,* 1992.), vaccinia CrmA protein (Ray, *et al.*), simian virus 40 (SV40) T antigen (McCarthy, *et al.*, 1994), human papillomavirus No. 16 (HPV 16) E6 protein (Pan and Griep, 1994), Epstein-Barr virus BHRF1 protein (Henderson, *et al.*, 1993) and human cytomegalovirus IE1 and IE2 gene products (Zhu, *et al.*, 1995). Herpes simplex virus 1 (HSV-1) encodes a protein, $\gamma_1$34.5, which blocks the phosphorylation of eIF-2α (Chou and Roizman, 1992).

The utility of proteins that are capable of inhibiting apoptosis are manifold. First, such proteins, or their corresponding genes, may be used to immortalize cell lines that otherwise would perish during culture. This makes possible not only the study of these cells, but also presents the option of growing these cells in large numbers in order to isolate protein species therefrom. Second, the identification of inhibitors of apoptosis and their function permits the possible intervention, in a clinical setting, when these proteins are interfering with normal programmed cell death, or apoptosis. This may be accomplished by providing an inhibitor or an antisense nucleic acid that interferes with the expression of a protein that interferes with apoptosis. Thus, the identification of novel proteins having these activities and uses provide important new tools for those working in this arena.

SUMMARY OF THE INVENTION

It is, therefore, an object of the present invention to provide methods for the use of U_S3 and its cognate gene U_S3 as inhibitors of apoptosis. The present invention further provides infected cell protein number 4, or ICP4, and its cognate gene α4, as inhibitors of apoptosis. In addition, it is an object of the present invention to provide methods for the use of agents that inhibit US3 protein function and gene expression to induce apoptosis in HSV infected cells. The present invention also provides methods for the use of agents that inhibit the apoptosis-induction function of US3 thereby to inhibiting apoptosis in virally infected cells. It is a further objective to provide methods of use of agents that inhibit ICP4 and/or α4 in order to induce apoptosis in HSV infected cells. It also is an object of the present invention to provide methods for the identification of agents that inhibit the apoptosis-inhibiting function of ICP4.

In satisfying these goals, there is provided a method for blocking apoptosis of a cell comprising the step of providing to the cell an HSV U_S3 polypeptide or an HSV U_S3 gene. The U_S3 gene may be contained in an expression vector and, further, under the control of a promoter active in eukaryotic cells. One such promoter is the a tetracycline controlled promoter. The expression vector further comprises a selectable marker and/or further comprises a gene encoding a second polypeptide under the transcriptional control of a promoter active in eukaryotic cells.

The method may further comprise the step of providing to the cell an HSV ICP4 polypeptide or an HSV α4 gene. The α4 gene may be contained in an expression vector and, further, under the control of a promoter active in eukaryotic cells.

In another embodiment, there is provided a method for inducing apoptosis in a cell infected with HSV comprising the step of administering to said cell an agent that inhibits HSV U_S3 function in said cell. The agent may inhibit transcription or translation of an HSV U_S3 gene or transcript or may bind to an HSV U_S3 polypeptide. The reagent may be an antisense HSV U_S3 construct an antibody that binds immunologically to an HSV U_S3 polypeptide. Particular antisense constructs are oligonucleotides that hybridizes to a 5'-untranslated region for an HSV U_S3 gene or a translational start site for an HSV U_S3 transcript. Particular antibodies are polyclonal sera against U_S3 or a monoclonal antibody against U_S3.

In another embodiment, the method further comprising the step of administering to said cell an agent that inhibits HSV ICP4 function in said cell.. The agent may inhibit transcription or translation of an HSV α4 gene or transcript or may bind to an HSV ICP4 polypeptide. The reagent may be an antisense HSV α4 construct an antibody that binds immunologically to an HSV ICP4 polypeptide. Particular antisense constructs are oligonucleotides that hybridizes to a 5'-untranslated region for an HSV α4 gene or a translational start site for an HSV α4 transcript. Particular antibodies are polyclonal sera against ICP4 or a monoclonal antibody against ICP4. The agent that inhibits ICP4 function may be administered prior to, concurrently with or after the administration of the agent that inhibits U_S3 function.

In yet another embodiment, there is provided a method for treating a subject with an HSV infection comprising the step of inhibiting HSV US3 function. The inhibition may comprise providing to the subject a first pharmaceutical composition comprising an HSV U_S3

antisense construct or a monoclonal antibody that binds immunologically to an HSV U_S3 polypeptide. The first pharmaceutical composition may be applied topically to HSV infected cells in said patient. The method may further comprise the step of providing to said subject a second pharmaceutical composition comprising a conventional anti-HSV agent, such as acyclovir. Acyclovir is delivered via a route selected from the group consisting of topically, orally and intravenously.

In a further aspect of the present invention the method for treating a subject with an HSV infection may further comprise the step of inhibiting HSV ICP4 function. The inhibition may comprise providing to the subject a first pharmaceutical composition comprising an HSV α4 antisense construct or a monoclonal antibody that binds immunologically to an HSV ICP4 polypeptide. The first pharmaceutical composition may be applied topically to HSV infected cells in said patient. The method may further comprise the step of providing to said subject a second pharmaceutical composition comprising a conventional anti-HSV agent, such as acyclovir. Acyclovir is delivered via a route selected from the group consisting of topically, orally and intravenously.

In yet still another embodiment, there is provided a screening method for compounds having inhibitory activity against HSV U_S3 polypeptide-induced inhibition of apoptosis comprising the steps of (a) providing a first cell comprising an HSV US3 gene under the control of an HSV immediate early promoter; (b) infecting said first cell with a herpes simplex virus that lacks a functional U_S3 gene; (c) contacting said first cell with a test compound; (d) incubating said first cell under conditions permitting viral replication; and (e) comparing the cell pathology of said first cell following incubation with the cell pathology of a second cell that lacks an HSV U_S3 gene following infection with said herpes simplex virus and the cell pathology of a third cell comprising an HSV U_S3 gene under the control of an HSV immediate early promoter following infection with said herpes simplex virus but in the absence of said test compound. Cell pathology comprises condensation of chromatin, obliteration of nuclear membranes, vacuolization, cytoplasmic blebbing and DNA fragmentation.

The screening method may employ a cell line which contains an integrated copy of a wild-type HSV U_S3 gene under the control of an α4 promoter and a herpes simplex virus that lacks a functional HSV U_S3 gene has a deletion in both copies of the virally-encoded U_S3

genes. For example, the herpes virus may carry a temperature sensitive mutation in both copies of the virally-encoded U_S3 gene; incubation is at 39.5°C.

Another screening method for compounds having inhibitory activity against HSV U_S3-induced inhibition of apoptosis comprises the steps of (a) providing a first cell comprising an HSV U_S3 gene under the control of an inducible promoter; (b) inducing transcription from said promoter; (c) contacting said first cell with a test compound; (d) incubating said first cell under conditions expression of an HSV U_S3 polypeptide; and (e) comparing the cell pathology of said first cell following incubation with the cell pathology of a second cell not having an HSV U_S3 gene following induction and the cell pathology of a third cell comprising an HSV U_S3 gene under the control of an inducible promoter following induction but in the absence of said test compound.

Yet another screening method for compounds having inhibitory activity against HSV U_S3 polypeptide-induced inhibition of apoptosis comprises the steps of (a) providing a first cell; (b) infecting said first cell with a herpes simplex virus; (c) contacting said first cell with a test compound; (d) incubating said first cell under conditions permitting viral replication; and (e) comparing the cell pathology of said first cell following incubation with the cell pathology of a second cell treated with said test compound alone and the cell pathology of a third cell following infection with said herpes simplex virus but in the absence of said test compound.

In still yet a further embodiment, there is provided a method for expressing a polypeptide in a cell comprising the steps of (a) contacting a cell with a herpes virus vector encoding said polypeptide; (b) contacting said cell with an agent that blocks the transactivating function of U_S3 but does not block the apoptosis inhibiting function of U_S3.

In yet still another embodiment, there is provided a screening method for compounds having inhibitory activity against HSV ICP4 polypeptide-induced inhibition of apoptosis comprising the steps of (a) providing a first cell comprising an HSV α4 gene under the control of an HSV immediate early promoter; (b) infecting said first cell with a herpes simplex virus that lacks a functional α4 gene; (c) contacting said first cell with a test compound; (d) incubating said first cell under conditions permitting viral replication; and (e) comparing the cell pathology of said first cell following incubation with the cell pathology of a second cell that lacks an HSV α4 gene following infection with said herpes simplex virus and the cell

pathology of a third cell comprising an HSV α4 gene under the control of an HSV immediate early promoter following infection with said herpes simplex virus but in the absence of said test compound. Cell pathology comprises condensation of chromatin, obliteration of nuclear membranes, vacuolization, cytoplasmic blebbing and DNA fragmentation.

The screening method may employ a cell line which contains an integrated copy of a wild-type HSV α4 gene under the control of an α4 promoter and a herpes simplex virus that lacks a functional HSV α4 gene has a deletion in both copies of the virally-encoded α4 genes. For example, the herpes virus may carry a temperature sensitive mutation in both copies of the virally-encoded α4 gene; incubation is at 39.5°C.

Another screening method for compounds having inhibitory activity against HSV ICP4-induced inhibition of apoptosis comprises the steps of (a) providing a first cell comprising an HSV α4 gene under the control of an inducible promoter; (b) inducing transcription from said promoter; (c) contacting said first cell with a test compound; (d) incubating said first cell under conditions expression of an HSV ICP4 polypeptide; and (e) comparing the cell pathology of said first cell following incubation with the cell pathology of a second cell not having an HSV α4 gene following induction and the cell pathology of a third cell comprising an HSV α4 gene under the control of an inducible promoter following induction but in the absence of said test compound.

Yet another screening method for compounds having inhibitory activity against HSV ICP4 polypeptide-induced inhibition of apoptosis comprises the steps of (a) providing a first cell; (b) infecting said first cell with a herpes simplex virus; (c) contacting said first cell with a test compound; (d) incubating said first cell under conditions permitting viral replication; and (e) comparing the cell pathology of said first cell following incubation with the cell pathology of a second cell treated with said test compound alone and the cell pathology of a third cell following infection with said herpes simplex virus but in the absence of said test compound.

In still yet a further embodiment, there is provided a method for expressing a polypeptide in a cell comprising the steps of (a) contacting a cell with a herpes virus vector encoding said polypeptide; (b) contacting said cell with an agent that blocks the transactivating function of ICP4 but does not block the apoptosis inhibiting function of ICP4.

The present invention also provides a method for the identification of a candidate substance that is a modulator of US3-kinase activity comprising the steps of (a) providing an enzyme composition comprising U_S3 protein kinase and a threonine/serine protein substrate; (b) contacting the composition with the candidate substance under conditions that allow phosphorylation; (c) determining the phosphorylation of the threonine/serine substrate; and (d) comparing the phosphorylation of the threonine/serine substrate with the phosphorylation of the threonine/serine substrate in the absence of a candidate inhibitor substance; wherein an increase in phosphorylation is indicative of the candidate substance being a stimulator of phosphorylation and a decrease in phosphorylation of the threonine/serine substrate is indicative of the candidate being an inhibitor of phosphorylation.

The present invention further provides an inhibitor of U_S3-kinase activity identified according to a method comprising the steps of (a) providing an enzyme composition comprising U_S3 protein kinase and a threonine/serine protein substrate; (b) contacting the composition with the candidate substance under conditions that allow phosphorylation; (c) determining the phosphorylation of the threonine/serine substrate; and (d) comparing the phosphorylation of the threonine/serine substrate with the phosphorylation of the threonine/serine substrate in the absence of a candidate inhibitor substance; wherein a decrease in phosphorylation of the threonine/serine substrate is indicative of the candidate being an inhibitor of phosphorylation.

In another embodiments there is provided a stimulator of U_S3-kinase activity identified according to a method comprising the steps of (a) providing an enzyme composition comprising U_S3 protein kinase and a threonine/serine protein substrate; (b) contacting said composition with said candidate substance under conditions that allow phosphorylation; (c) determining the phosphorylation of said threonine/serine substrate; and (d) comparing the phosphorylation of said threonine/serine substrate with the phosphorylation of said threonine/serine substrate in the absence of a candidate inhibitor substance; wherein an increase in phosphorylation is indicative of said candidate substance being a stimulator of phosphorylation.

Other objects, features and advantages of the present invention will become apparent from the following detailed description. It should be understood, however, that the detailed

description and the specific examples, while indicating preferred embodiments of the invention, are given by way of illustration only, since various changes and modifications within the spirit and scope of the invention will become apparent to those skilled in the art from this detailed description.

The following drawings form part of the present specification and are included to further demonstrate certain aspects of the present invention. The invention may be better understood by reference to one or more of these drawings in combination with the detailed description of specific embodiments presented herein.

FIG. 1: Diagrammatic representation of the HSV-1 genome showing the location of the α4 and U_L29 genes encoding ICP4 and ICP8, respectively. The reiterated sequences (open rectangles) flanking the unique short (U_S) and unique long (U_L) sequences (thin lines) and the location and direction of genes are as shown. Because the α4 gene maps within inverted repeats flanking the U_L, it is present in two copies per genome. The hatched lines within the rectangles indicates the position of the sequences deleted from the d120 mutant (DeLuca, *et al.*, 1985).

FIG. 2. Photograph of an agarose gel stained with ethidium bromide. Vero cells were mock-infected (lane 1), infected with HSV-1(F) (lane 2), HSV-1 d120 mutant (lane 3), HSV-1 120FR mutant (lane 4), or HSV-1 120KR mutant (lane 5). 30 hr post infection, 2×10^6 cells per sample were collected, washed in PBS, lysed in a solution containing 10 mM Tris-HCl, pH 8.0, 10 mM EDTA, and 0.5% Triton X-100, and centrifuged at 12,000 rpm for 25 min in an Eppendorf microcentrifuge to pellet chromosomal DNA. Supernatant fluids were digested with 0.1 mg RNase A per ml at 37°C for 1 hr and then for 2 hr with 1 mg proteinase K per ml at 50°C in the presence of 1% sodium dodecylsulphase (SDS), extracted with phenol and chloroform, and precipitated in cold ethanol and subjected to electrophoresis on horizontal 1.5% agarose gels containing 5 μg of ethidium bromide per ml. DNA was visualized by UV light transillumination. Photographs were taken with the aid of a computer-assisted image processor (Eagle Eye II, Stratagene).

FIG. 3. Photograph of an agarose gel stained with ethidium bromide. Vero cells were mock-infected (lane 1), infected with HSV-1 120FR mutant (lane 2), wild-type HSV-1(F) (lane

3), HSV-1 R325 mutant (lane 4), or HSV-1 R7041 mutant (lane 5). Cells were harvested and processed as described in the legend to FIG. 2.

FIG. 4. Photograph of an agarose gel stained with ethidium bromide. Vero cells were infected with HSV-1(F) (lane 1), HSV-1 120FR mutant (lane 2), HSV-1 R7041 mutant (lane 3), HSV-1 R7306 mutant (lane 4), or double-infected with HSV-1 120FR and R7041 mutants (lane 5), HSV-1 d120 and 120FR mutants (lane 6), or HSV-1 120FR and R7306 mutants (lane 7). Cells were harvested and processed as described in the legend to FIG. 2.

FIG. 5. BHK-C13 cells were infected at a multiplicity of 10 pfu/cell with the indicated viruses. At 13 hr post infection cells were incubated in phosphate-free medium for 1 hr and then labeled with 32Pi for 4 hr. Cell lysates were electrophoretically separated in SDS polyacrylamide gels and electrically transferred onto nitrocellulose filters. Filters were exposed to film or probed with a U_L34 antibody.

DETAILED DESCRIPTION OF THE INVENTION

The identification of novel proteins having apoptotic activities and uses will provide important new tools for those working with the challenge of treatment and prevention of HSV infection. The present invention provides methods for the use of U_S3 gene and its gene product as inhibitors of apoptosis.

The herpes simplex virus 1 (HSV-1) mutant, which lacks the major regulatory gene designated α4, induced apoptosis, whereas in cells infected with wild-type virus, apoptosis did not ensue (Leopardi and Roizman, 1996). The inventors also reported that wild type virus blocked apoptosis induced by thermal shock. Recent studies by Koyama and Miwa (1997) and also in the inventors' laboratory demonstrated that the virus also blocks the induction of apoptosis by osmotic shock. These studies suggested that a functional α4 gene was necessary to block apoptosis but did not address the question of sufficiency. This led the inventors to design several studies that included the rescue of the deleted α4 genes in the mutant virus.

The inventors discovered that the mutant lacking the α4 gene, HSV-1(KOS)d120, had a secondary mutation in the gene U_S3 specifying a protein kinase. Thus, the inventors have discovered that a functional U_S3 is required in order to block apoptosis. This important finding

may now be exploited in the inhibition of apoptosis, the induction of apoptosis, as described herein below. Further, the present invention allows for the provision of methods for the use of agents that inhibit U_S3 and the U_S3 gene in order to induce apoptosis in HSV infected cells and for the identification of agents that inhibit the apoptosis-inhibiting function of ICP4. These and other related aspects of the present invention are described in further detail herein below.

I. *Herpes Simplex Virus*

Herpes simplex viruses, designated with subtypes 1 and 2, are enveloped viruses that are among the most common infectious agents encountered by humans, infecting millions of human subjects worldwide. These viruses cause a broad spectrum of disease which ranges from relatively insignificant to severe and life-threatening. The clinical outcome of herpes infections is dependent upon early diagnosis and prompt initiation of antiviral therapy. Despite some successful efforts in treating HSV infectious, dermal and epidermal lesion often recur, and HSV infections of neonates and infections of the brain are associated with high morbidity and mortality.

The large, complex, double-stranded DNA genome encodes dozens of different gene products, some of which derive from spliced transcripts. In addition to virion and envelope structural components, the virus encodes numerous other proteins including a protease, a ribonucleotides reductase, a DNA polymerase, a ssDNA binding protein, a helicase/primase, a DNA dependent ATPase, a dUTPase and others.

HSV genes form several groups whose expression is coordinately regulated and sequentially ordered in a cascade fashion (Honess and Roizman, 1974; Honess and Roizman 1975; Roizman and Sears, 1996). The expression of α genes, the first set of genes to be expressed after infection, is enhanced by the virion protein number 16, or α-transinducing factor (Post *et al.*, 1981; Batterson and Roizman, 1983; Campbell, *et al.*, 1984). The expression of β genes requires functional α gene products, most notably ICP4, which is encoded by the $\alpha 4$ gene (DeLuca *et al.*, 1985). γ genes, a heterogeneous group of genes encoding largely virion structural proteins, require the onset of viral DNA synthesis for optimal expression (Holland *et al.*, 1980).

In line with the complexity of the genome, the life cycle of HSV is quite involved. In addition to the lytic cycle, which results in synthesis of virus particles and, eventually, cell death, the virus has the capability to enter a latent state in which the genome is maintained in neural ganglia until some as of yet undefined signal triggers a recurrence of the lytic cycle.

II. *The ICP4 Polypeptide*

As stated above, the expression of β genes is regulated in a major fashion by ICP4 (DeLuca *et al.*, 1985), and therefore this gene has a distinct effect on viral DNA synthesis. γ genes, a heterogeneous group of genes encoding largely virion structural proteins, require the onset of viral DNA synthesis for optimal expression (Honess and Roizman, 1975; Holland *et al.*, 1980). ICP4 plays a key role in this process - cells infected with viruses carrying temperature sensitive mutations in the α4 gene and maintained at nonpermissive temperatures express largely α proteins (Dixon and Shaffer, 1980). Furthermore, studies involving shift-up of infected cells from permissive to nonpermissive temperatures have confirmed a key role for ICP4 throughout the viral reproductive cycle.

ICP4 acts both as a transactivator and as a repressor (Roizman and Sears, 1996). The response elements for the repressor functions of ICP4 are high affinity binding sites located in the proximity of transcription initiation sites of the genes repressed by the protein (Kristie and Roizman, 1986a, Kristie and Roizman, 1986b, Faber and Wilcox, 1986, Muller, 1987, Michael and Roizman, 1989, Michael and Roizman, 1993). The strength of repression is dependent on both the distance and stereoaxial alignment with the TATA box (Leopardi *et al.*, 1995, Kuddus *et al.*, 1995). Low affinity sites for binding of ICP4 have also been documented, but their function is less well understood (Kristie and Roizman, 1986a, Kristie and Roizman, 1986b, Michael *et al.*, 1988). The response elements thought to act in the transactivation of viral genes by ICP4 are not known, but mutations in ICP4 may affect repression and activation independently of each other (Shepard and DeLuca, 1991).

Thus, the repressor function of ICP4 is associated with the presence of high affinity sites located at or near the transcription initiation sites of several genes. The transactivating function has not been associated to specific binding to DNA although it has been shown that late viral gene expression requires the association of ICP4 with nascent viral DNA and several host (*e.g.*,

RNA polymerase II, Epstein Barr virus small nuclear RNA associated host protein) proteins and ICP22, a viral transcriptional factor (Leopardi *et al.*, 1997). Extensive studies with deletion mutants exemplified by HSV-1(KOS)d120 led DeLuca *et al.* (1985) to map distinct domains within the ICP4 proteins. Studies in the inventors' laboratory have shown that ICP4 is extensively modified post translationally by phosphorylation, ADP(ribosyl)ation, and nucleotidylylation (Roizman and Sears, 1996). The expectation is that these modifications are not random effects by existing enzymes but that each post translational modification enhances or inactivates a specific function of the protein.

III. *The U_S3 Polypeptide*

HSV encodes two protein kinases expressed by the genes U_S3 and U_L13, respectively (reviewed in Roizman and Sears, 1996). Whereas U_L13 is packaged in the virion, U_S3 is not. Not all substrates of the U_S3 are known (Purves *et al.*, 1986; 1987; Leader *et al.*, 1991). The major substrate of U_S3 protein kinase is an intrinsic membrane protein exposed on the surface of infected cells and encoded by the U_L34 gene (Purves *et al.*, 1991; 1992). The U_L34 is phosphorylated by more than one kinase (Purves 1991, 1992). In the absence of U_S3 protein kinase, the U_L34 protein has an apparent M_r of 33,000 and associates with several cellular phosphoproteins whereas in wild type infected cells U_L34 has an apparent M_r of 30,000 and does not exhibit an association with the host proteins (Purves *et al.*, 1992).

The U_S3 protein kinase phosphorylates theronine/serine in the consensus sequence RRR-R/X-S/T-R/Y (SEQ ID NO:6) (Purves *et al.*, 1986; Leader *et al.*, 1991). In the case of U_L34, this was verified by mutagenesis of the threonine codon in the sequence encoding RRRRTRRSRE (SEQ ID NO:5) (Purves *et al.*, 1991; 1992). The apparent M_r of the mutant U_L34 protein was 33,000, that is, corresponding to the apparent M_r of the U_L34 protein in cells infected with a virus lacking the U_S3 gene.

The inventors predict that U_S3 blocks apoptosis by phosphorylating one or more proteins. As stated above, for ICP4 to be functional it needs to be phosphorylated, this leads the inventors to believe that one of the substrates of U_S3 protein kinase is ICP4 itself, among other, as yet unidentified, phosphoproteins.

The involvement of U_S3 protein kinase in blocking of apoptosis induced by infection, thermal or osmotic shocks suggests that HSV differs from other viruses in the mechanism by which it blocks the apoptosis as a host response to infection. Thus, U_S3 may be used to in control apoptosis independent of viral infection.

Accordingly, the present invention describes a novel functional aspect of the protein kinase U_S3 that was previously undisclosed is its ability to inhibit apoptosis. Further, the invention describes a novel functional aspect of ICP4 as an inhibitor of apoptosis. Apoptosis, or programmed cell death, is characterized by certain cellular events, including nuclear condensation, DNA fragmentation, cytoplasmic membrane blebbing and, ultimately, irreversible cell death. Apoptosis is an energy-dependent event. For the purposes of this application, apoptosis will be defined as inducing one or more of these events. Thus, use of the term "U_S3" in this application encompasses polypeptides having the anti-apoptosis function of U_S3. These need not be wild-type U_S3. Likewise, the use of the term "ICP4" in this application encompasses polypeptides having the anti-apoptosis function of ICP4 and it need not be wild-type ICP4.

This functional attribute is manifested, for example, in U_S3's or ICP4's ability, alone or in combination with one another, to protect cells from apoptosis triggered by modification of cellular physiology by other viral genes. This observation permits utilization of U_S3 or ICP4 alone or in combination with each other, in a number of ways that could not have been predicted from the prior art. For example, according to the present invention, the production of HSV vectors or recombinant proteins from HSV vectors can be enhanced by increasing the apoptosis inhibiting function of U_S3 or ICP4 or both. When cells are infected with HSV, premature cell death can limit the titer of virus produced or the amount of recombinant protein synthesized. Similarly, U_S3 and ICP4 may prolong the life of the cells expressing human or animal genes introduced into cells by viral vectors in order to correct genetic defect. If the cell can be sustained longer, the titer of the virus stocks and the amount of protein should increase.

U_S3 and ICP4 may be obtained according to various standard methodologies that are known to those of skill in the art. For example, antibodies specific for U_S3 or ICP4 may be used in immunoaffinity protocols to isolate the respective polypeptide from infected cells, in particular, from infected cell lysates. Antibodies are advantageously bound to supports, such as

columns or beads, and the immobilized antibodies can be used to pull the U_S3 or IPC4 target out of the cell lysate.

Alternatively, expression vectors, rather than viral infections, may be used to generate the polypeptide of interest. A wide variety of expression vectors may be used, including viral vectors. The structure and use of these vectors is discussed further, below. Such vectors may significantly increase the amount of U_S3 and/or ICP4 protein in the cells, and may permit less selective purification methods such as size fractionation (chromatography, centrifugation), ion exchange or affinity chromatograph, and even gel purification. Alternatively, the expression vector may be provided directly to target cells, again as discussed further, below.

U_S3, according to the present invention, may advantageously be cleaved into fragments for use in further structural or functional analysis, or in the generation of reagents such as U_S3-related polypeptides and U_S3-specific antibodies. This can be accomplished by treating purified or unpurified U_S3 with a peptidase such as endoproteinase glu-C (Boehringer, Indianapolis, IN). Treatment with CNBr is another method by which U_S3 fragments may be produced from natural U_S3. Recombinant techniques also can be used to produce specific fragments of U_S3. It may be that the phosphorylating and apoptosis-inhibiting functions of U_S3 reside in distinct domains of the protein. If such is the case, the ability to make domain-specific reagents now has significance. For example, the ability to provide an apoptosis-inhibiting U_S3 fragment that does not phosphorylate viral genes may prove to be effective in extending the life of neurons expressing compensatory or therapeutic genes from a viral vector.

Likewise ICP4, may advantageously be cleaved into fragments for use in further structural or functional analysis, or in the generation of reagents such as ICP4-related polypeptides and ICP4-specific antibodies. Because the transactivating and apoptosis-inhibiting functions of ICP4 appear to reside in distinct domains, the ability to make domain-specific reagents now has significance. For example, the ability to provide an apoptosis-inhibiting ICP4 fragment that does not transactivate viral genes may prove to be effective in extending the life of neurons expressing compensatory or therapeutic genes from a viral vector.

It is expected that changes may be made in the sequence of U_S3 or ICP4 while retaining a molecule having the structure and function of the natural U_S3 or ICP4 respectively. For example, certain amino acids may be substituted for other amino acids in a protein structure without

appreciable loss of interactive capacity with structures such as, for example, substrate-binding regions. These changes are termed "conservative" in the sense that they preserve the structural and, presumably, required functional qualities of the starting molecule. The importance of U_S3 variants is highlighted by the observation, discussed in the examples, that mutants lacking the α4 gene also have an additional mutation in the US3 gene making it non-functional in apoptosis. The mutation in U_S3 was silent and would not have affected the studies carried out by DeLuca *et al.* (1985) since viral gene expression, including that of U_S3, requires a functional α4 gene and apoptosis is a very late event. Even if a rescue had been done, it would not have detected the mutation in U_S3 unless the infected cell were probed for the U_S3 function.

The importance of ICP4 variants is highlighted by the observation that temperature sensitive (*ts*) mutants of ICP4 exist that are impaired in their ability to transactivate viral genes at elevated temperatures (above about 39°C), but retain the apoptosis inhibiting function associated with this polypeptide. Further exploration of this dichotomy should reveal significant information on the regions in which these functions lie. It has been shown that the transactivation domain of ICP4 lies between about residues 100 and 200, the DNA-binding domains lies between about residues 300 and 500, the nuclear localization domain lies between about residues 700 and 750 and the transactivation domain lies between about residues 750 and 1298.

Conservative amino acid substitutions generally are based on the relative similarity of the amino acid side-chain substituents, for example, their hydrophobicity, hydrophilicity, charge, size, and the like. An analysis of the size, shape and type of the amino acid side-chain substituents reveals that arginine, lysine and histidine are all positively charged residues; that alanine, glycine and serine are all a similar size; and that phenylalanine, tryptophan and tyrosine all have a generally similar shape. Therefore, based upon these considerations, arginine, lysine and histidine; alanine, glycine and serine; and phenylalanine, tryptophan and tyrosine; are defined herein as equivalent.

In making such changes, the hydropathic index of amino acids also may be considered. Each amino acid has been assigned a hydropathic index on the basis of their hydrophobicity and charge characteristics, these are: isoleucine (+4.5); valine (+4.2); leucine (+3.8); phenylalanine (+2.8); cysteine/cystine (+2.5); methionine (+1.9); alanine (+1.8); glycine (-0.4); threonine (-0.7);

serine (-0.8); tryptophan (-0.9); tyrosine (-1.3); proline (-1.6); histidine (-3.2); glutamate (-3.5); glutamine (-3.5); aspartate (-3.5); asparagine (-3.5); lysine (-3.9); and arginine (-4.5).

The importance of the hydropathic amino acid index in conferring interactive biological function on a protein is generally understood in the art (Kyte & Doolittle, 1982). It is known that certain amino acids may be substituted for other amino acids having a similar hydropathic index or score and still retain a similar biological activity. In making changes based upon the hydropathic index, the substitution of amino acids whose hydropathic indices are within ±2 is preferred, those which are within ±1 are particularly preferred, and those within ±0.5 are even more particularly preferred.

It also is understood in the art that the substitution of like amino acids can be made effectively on the basis of hydrophilicity, particularly where the polypeptide created is intended for use in immunological embodiments, as in the present case. U.S. Patent 4,554,101, incorporated herein by reference, states that the greatest local average hydrophilicity of a protein, as governed by the hydrophilicity of its adjacent amino acids, correlates with its immunogenicity and antigenicity, *i.e.*, with a biological property of the protein.

As detailed in U.S. Patent 4,554,101, the following hydrophilicity values have been assigned to amino acid residues: arginine (+3.0); lysine (+3.0); aspartate (+3.0 ± 1); glutamate (+3.0 ± 1); serine (+0.3); asparagine (+0.2); glutamine (+0.2); glycine (0); threonine (-0.4); proline (-0.5 ± 1); alanine (-0.5); histidine (-0.5); cysteine (-1.0); methionine (-1.3); valine (-1.5); leucine (-1.8); isoleucine (-1.8); tyrosine (-2.3); phenylalanine (-2.5); tryptophan (-3.4).

In making changes based upon similar hydrophilicity values, the substitution of amino acids whose hydrophilicity values are within ±2 is preferred, those which are within ±1 are particularly preferred, and those within ±0.5 are even more particularly preferred.

Numerous scientific publications have been devoted to the prediction of secondary structure, and to the identification of epitopes, from analyses of amino acid sequences (Chou & Fasman, 1974a,b; 1978a,b; 1979). Any of these may be used, if desired, to supplement the teachings of Hopp in U.S. Patent 4,554,101. Moreover, computer programs are currently available to assist with predicting antigenic portions and epitopic core regions of proteins. Examples include those programs based upon the Jameson-Wolf analysis (Jameson & Wolf,

1988; Wolf *et al.*, 1988), the program PepPlot® (Brutlag *et al.*, 1990; Weinberger *et al.*, 1985), and other new programs for protein tertiary structure prediction (Fetrow & Bryant, 1993).

Two designations for amino acids are used interchangeably throughout this application, as is common practice in the art. Alanine = Ala (A); Arginine = Arg (R); Aspartate = Asp (D); Asparagine = Asn (N); Cysteine = Cys (C); Glutamate = Glu (E); Glutamine = Gln (Q); Glycine = Gly (G); Histidine = His (H); Isoleucine = Ile (I); Leucine = Leu (L); Lysine = Lys (K); Methionine = Met (M); Phenylalanine = Phe (F); Proline = Pro (P); Serine = Ser (S); Threonine= Thr (T); Tryptophan = Trp (W); Tyrosine = Tyr (Y); Valine= Val (V).

In addition to the peptidyl compounds described herein, the inventors also contemplate that other sterically similar compounds may be formulated to mimic the key portions of the peptide structure, called peptidomimetics. Mimetics are peptide-containing molecules which mimic elements of protein secondary structure. See, for example, Johnson *et al.* (1993). The underlying rationale behind the use of peptide mimetics is that the peptide backbone of proteins exists chiefly to orient amino acid side chains in such a way as to facilitate molecular interactions, such as those of receptor and ligand.

Successful applications of the peptide mimetic concept have thus far focused on mimetics of β-turns within proteins. Likely β-turn structures within U_S3 and ICP4 can be predicted by computer-based algorithms as discussed above. Once the component amino acids of the turn are determined, mimetics can be constructed to achieve a similar spatial orientation of the essential elements of the amino acid side chains, as discussed in Johnson *et al.*, *supra.*

IV. *Nucleic Acids Encoding U_S3 and ICP4*

Also contemplated by the present invention are nucleic acids encoding U_S3 and ICP4. The gene for US3 is given in SEQ ID NO:3. The gene for ICP4 is known as α4 (SEQ ID NO:1). The amino acid sequences for U_S3 and ICP4 encoded by these genes are given in SEQ ID NO:4 and SEQ ID NO:2, respectively. The full length genomic sequence of HSV is known and can be found in Genbank (Accession No. x14112); ICP4 is encoded by identical diploid genes inverted relative to each other, their coding sequences are located from nucleotide 131, 128 to 127, 232 and 147,104 to 151, 000. The US3 protein coding sequence is from nucleotide 135, 222 to 136, 667. Because of the degeneracy of the genetic code, many other nucleic acids also may encode a given

U_S3 or a given ICP4. For example, four different three-base codons encode the amino acids alanine, glycine, proline, threonine and valine, while six different codons encode arginine, leucine and serine. Only methionine and tryptophan are encoded by a single codon. A table of amino acids and the corresponding codons is presented herein for use in such embodiments.

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TABLE 1

Amino Acids			Codons					
Alanine	Ala	A	GCA	GCC	GCG	GCU		
Cysteine	Cys	C	UGC	UGU				
Aspartic acid	Asp	D	GAC	GAU				
Glutamic acid	Glu	E	GAA	GAG				
Phenylalanine	Phe	F	UUC	UUU				
Glycine	Gly	G	GGA	GGC	GGG	GGU		
Histidine	His	H	CAC	CAU				
Isoleucine	Ile	I	AUA	AUC	AUU			
Lysine	Lys	K	AAA	AAG				
Leucine	Leu	L	UUA	UUG	CUA	CUC	CUG	CUU
Methionine	Met	M	AUG					
Asparagine	Asn	N	AAC	AAU				
Proline	Pro	P	CCA	CCC	CCG	CCU		
Glutamine	Gln	Q	CAA	CAG				
Arginine	Arg	R	AGA	AGG	CGA	CGC	CGG	CGU

TABLE 1 - Continued

Amino Acids			Codons					
Serine	Ser	S	AGC	AGU	UCA	UCC	UCG	UCU
Threonine	Thr	T	ACA	ACC	ACG	ACU		
Valine	Val	V	GUA	GUC	GUG	GUU		
Tryptophan	Trp	W	UGG					
Tyrosine	Tyr	Y	UAC	UAU				

In order to generate any nucleic acid encoding U_S3 or ICP4, one need only refer to the preceding codon table. Substitution of the natural codon with any codon encoding the same amino acid will result in a distinct nucleic acid that encodes U_S3 or ICP4 or a variant thereof. As a practical matter, this can be accomplished by site-directed mutagenesis of an existing U_S3 or α4 gene or *de novo* chemical synthesis of one or more nucleic acids.

The preceding observations regarding codon selection, site-directed mutagenesis and chemical synthesis apply with equal force to the discussion of substitutional mutants in the section of peptides. Normally, substitutional mutants are generated by site-directed changes in the nucleic acid designed to alter one or more codons of the coding sequence.

In order to express an U_S3 polypeptide, or an antisense U_S3 transcript, it is necessary to provide an U_S3 gene in an expression vehicle. Similarly to express an ICP4 polypeptide, or an antisense α4 transcript, it is necessary to provide an α4 gene in an expression vehicle. The appropriate nucleic acid can be inserted into an expression vector by standard subcloning techniques. For example, an *E. coli* or baculovirus expression vector is used to produce recombinant polypeptide *in vitro*. The manipulation of these vectors is well known in the art. In one embodiment, the protein is expressed as a fusion protein with β-gal, allowing rapid affinity purification of the protein. Examples of such fusion protein expression systems are the glutathione S-transferase system (Pharmacia, Piscataway, NJ), the maltose binding protein

system (NEB, Beverley, MA), the FLAG system (IBI, New Haven, CT), and the 6xHis system (Qiagen, Chatsworth, CA).

Some of these fusion systems produce recombinant protein bearing only a small number of additional amino acids, which are unlikely to affect the functional capacity of the recombinant protein. For example, both the FLAG system and the 6xHis system add only short sequences, both of which are known to be poorly antigenic and which do not adversely affect folding of the protein to its native conformation. Other fusion systems produce proteins where it is desirable to excise the fusion partner from the desired protein. In another embodiment, the fusion partner is linked to the recombinant protein by a peptide sequence containing a specific recognition sequence for a protease. Examples of suitable sequences are those recognized by the Tobacco Etch Virus protease (Life Technologies, Gaithersburg, MD) or Factor Xa (New England Biolabs, Beverley, MA).

Recombinant bacterial cells, for example *E. coli*, are grown in any of a number of suitable media, for example LB, and the expression of the recombinant polypeptide induced by adding IPTG to the media or switching incubation to a higher temperature. After culturing the bacteria for a further period of between 2 and 24 hours, the cells are collected by centrifugation and washed to remove residual media. The bacterial cells are then lysed, for example, by disruption in a cell homogenizer and centrifuged to separate the dense inclusion bodies and cell membranes from the soluble cell components. This centrifugation can be performed under conditions whereby the dense inclusion bodies are selectively enriched by incorporation of sugars such as sucrose into the buffer and centrifugation at a selective speed.

If the recombinant protein is expressed in the inclusion bodies, as is the case in many instances, these can be washed in any of several solutions to remove some of the contaminating host proteins, then solubilized in solutions containing high concentrations of urea (*e.g.* 8M) or chaotropic agents such as guanidine hydrochloride in the presence of reducing agents such as ß-mercaptoethanol or DTT (dithiothreitol).

Under some circumstances, it may be advantageous to incubate the polypeptide for several hours under conditions suitable for the protein to undergo a refolding process into a conformation which more closely resembles that of the native protein. Such conditions generally include low protein concentrations less than 500 µg/ml, low levels of reducing agent,

concentrations of urea less than 2 M and often the presence of reagents such as a mixture of reduced and oxidized glutathione which facilitate the interchange of disulphide bonds within the protein molecule.

The refolding process can be monitored, for example, by SDS-PAGE or with antibodies which are specific for the native molecule (which can be obtained from animals vaccinated with the native molecule isolated from parasites). Following refolding, the protein can then be purified further and separated from the refolding mixture by chromatography on any of several supports including ion exchange resins, gel permeation resins or on a variety of affinity columns.

In yet another embodiment, the expression system used is one driven by the baculovirus polyhedron promoter. The gene encoding the protein can be manipulated by standard techniques in order to facilitate cloning into the baculovirus vector. A preferred baculovirus vector is the pBlueBac vector (Invitrogen, Sorrento, CA). The vector carrying the gene of interest is transfected into *Spodoptera frugiperda* (Sf9) cells by standard protocols, and the cells are cultured and processed to produce the recombinant protein.

There also are a variety of eukaryotic vectors that provide a suitable vehicle in which recombinant polypeptide can be produced. HSV itself has been used in tissue culture to express a large number of exogenous genes as well as for high level expression of its endogenous genes. For example, the chicken ovalbumin gene has been expressed from HSV using an α promoter. Herz and Roizman (1983). The *lac*Z gene also has been expressed under a variety of HSV promoters.

In an alternative embodiment, the nucleic acids employed actually may encode antisense constructs that hybridize, under intracellular conditions, to an U_S3 nucleic acid or an α4 nucleic acid. The term "antisense construct" is intended to refer to nucleic acids, preferably oligonucleotides, that are complementary to the base sequences of a target DNA or RNA. Antisense oligonucleotides, when introduced into a target cell, specifically bind to their target nucleic acid and interfere with transcription, RNA processing, transport, translation and/or stability.

Antisense constructs may be designed to bind to the promoter and other control regions, exons, introns or even exon-intron boundaries of a gene. Antisense RNA constructs, or DNA encoding such antisense RNA's, may be employed to inhibit gene transcription or translation or both within a host cell, either *in vitro* or *in vivo*, such as within a host animal, including a human subject. Nucleic acid sequences which comprise "complementary nucleotides" are those which are capable of base-pairing according to the standard Watson-Crick complementarity rules. That is, that the larger purines will base pair with the smaller pyrimidines to form combinations of guanine paired with cytosine (G:C) and adenine paired with either thymine (A:T), in the case of DNA, or adenine paired with uracil (A:U) in the case of RNA. Inclusion of less common bases such as inosine, 5-methylcytosine, 6-methyladenine, hypoxanthine and others in hybridizing sequences does not interfere with pairing.

As used herein, the term "complementary" means nucleic acid sequences that are substantially complementary over their entire length and have very few base mismatches. For example, nucleic acid sequences of fifteen bases in length may be termed complementary when they have a complementary nucleotide at thirteen or fourteen positions with only a single mismatch. Naturally, nucleic acid sequences which are "completely complementary" will be nucleic acid sequences which are entirely complementary throughout their entire length and have no base mismatches.

Other sequences with lower degrees of homology also are contemplated. For example, an antisense construct which has limited regions of high homology, but also contains a non-homologous region (*e.g.*, a ribozyme) could be designed. These molecules, though having less than 50% homology, would bind to target sequences under appropriate conditions.

While all or part of the gene sequence of interest may be employed in the context of antisense construction, short oligonucleotides are easier to make and increase *in vivo* accessibility. However, both binding affinity and sequence specificity of an antisense oligonucleotide to its complementary target increases with increasing length. It is contemplated that antisense oligonucleotides of 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 25, 30, 35, 40, 45, 50, 60, 70, 80, 90, 100 or more base pairs will be used. One can readily determine whether a given antisense nucleic acid is effective at targeting of the corresponding host cell gene simply by testing the constructs *in vitro* to determine whether the endogenous gene's function is

affected or whether the expression of related genes having complementary sequences is affected.

In certain embodiments, one may wish to employ antisense constructs which include other elements, for example, those which include C-5 propyne pyrimidines. Oligonucleotides which contain C-5 propyne analogues of uridine and cytidine have been shown to bind RNA with high affinity and to be potent antisense inhibitors of gene expression (Wagner *et al.*, 1990).

Throughout this application, the term "expression construct" is meant to include any type of genetic construct containing a nucleic acid coding for a gene product in which part or all of the nucleic acid encoding sequence is capable of being transcribed. The transcript may be translated into a protein, but it need not be. Thus, in certain embodiments, expression includes both transcription of a gene and translation of a RNA into a gene product. In other embodiments, expression only includes transcription of the nucleic acid, for example, to generate antisense constructs.

In preferred embodiments, the nucleic acid is under transcriptional control of a promoter. A "promoter" refers to a DNA sequence recognized by the synthetic machinery of the cell, or introduced synthetic machinery, required to initiate the specific transcription of a gene. The phrase "under transcriptional control" means that the promoter is in the correct location and orientation in relation to the nucleic acid to control RNA polymerase initiation and expression of the gene.

The term promoter will be used here to refer to a group of transcriptional control modules that are clustered around the initiation site for RNA polymerase II. Much of the thinking about how promoters are organized derives from analyses of several viral promoters, including those for the HSV thymidine kinase (tk) and SV40 early transcription units. These studies, augmented by more recent work, have shown that promoters are composed of discrete functional modules, each consisting of approximately 7-20 bp of DNA, and containing one or more recognition sites for transcriptional activator or repressor proteins.

At least one module in each promoter functions to position the start site for RNA synthesis. The best known example of this is the TATA box, but in some promoters lacking a

TATA box, such as the promoter for the mammalian terminal deoxynucleotidyl transferase gene and the promoter for the SV40 late genes, a discrete element overlying the start site itself helps to fix the place of initiation.

Additional promoter elements regulate the frequency of transcriptional initiation. Typically, these are located in the region 30-110 bp upstream of the start site, although a number of promoters have recently been shown to contain functional elements downstream of the start site as well. The spacing between promoter elements frequently is flexible, so that promoter function is preserved when elements are inverted or moved relative to one another. In the tk promoter, the spacing between promoter elements can be increased to 50 bp apart before activity begins to decline. Depending on the promoter, it appears that individual elements can function either co-operatively or independently to activate transcription.

The particular promoter that is employed to control the expression of a nucleic acid is not believed to be critical, so long as it is capable of expressing the nucleic acid in the targeted cell. Thus, where a human cell is targeted, it is preferable to position the nucleic acid coding region adjacent to and under the control of a promoter that is capable of being expressed in a human cell. Generally speaking, such a promoter might include either a human or viral promoter. Preferred promoters include those derived from HSV, including the U_S3, or the $\alpha 4$ promoter. Another preferred embodiment is the tetracycline controlled promoter.

In various other embodiments, the human cytomegalovirus (CMV) immediate early gene promoter, the SV40 early promoter and the Rous sarcoma virus long terminal repeat can be used to obtain high-level expression of transgenes. The use of other viral or mammalian cellular or bacterial phage promoters which are well-known in the art to achieve expression of a transgene is contemplated as well, provided that the levels of expression are sufficient for a given purpose. Tables 2 and 3 list several elements/promoters which may be employed, in the context of the present invention, to regulate the expression of a transgene. This list is not intended to be exhaustive of all the possible elements involved in the promotion of transgene expression but, merely, to be exemplary thereof.

Enhancers were originally detected as genetic elements that increased transcription from a promoter located at a distant position on the same molecule of DNA. This ability to act over a large distance had little precedent in classic studies of prokaryotic transcriptional regulation.

Subsequent work showed that regions of DNA with enhancer activity are organized much like promoters. That is, they are composed of many individual elements, each of which binds to one or more transcriptional proteins.

The basic distinction between enhancers and promoters is operational. An enhancer region as a whole must be able to stimulate transcription at a distance; this need not be true of a promoter region or its component elements. On the other hand, a promoter must have one or more elements that direct initiation of RNA synthesis at a particular site and in a particular orientation, whereas enhancers lack these specificities. Promoters and enhancers are often overlapping and contiguous, often seeming to have a very similar modular organization.

Additionally any promoter/enhancer combination (as per the Eukaryotic Promoter Data Base EPDB) could also be used to drive expression of a transgene. Use of a T3, T7 or SP6 cytoplasmic expression system is another possible embodiment. Eukaryotic cells can support cytoplasmic transcription from certain bacterial promoters if the appropriate bacterial polymerase is provided, either as part of the delivery complex or as an additional genetic expression construct.

TABLE 2

PROMOTER
Immunoglobulin Heavy Chain
Immunoglobulin Light Chain
T-Cell Receptor
HLA DQ α and DQ ß
ß-Interferon
Interleukin-2
Interleukin-2 Receptor
MHC Class II 5
MHC Class II HLA-DRα
ß-Actin

TABLE 2 - Continued

PROMOTER
Muscle Creatine Kinase
Prealbumin (Transthyretin)
Elastase *I*
Metallothionein
Collagenase
Albumin Gene
α-Fetoprotein
τ-Globin
ß-Globin
c-fos
c-HA-ras
Insulin
Neural Cell Adhesion Molecule (NCAM)
$\alpha_{1\text{-Antitrypsin}}$
H2B (TH2B) Histone
Mouse or Type I Collagen
Glucose-Regulated Proteins (GRP94 and GRP78)
Rat Growth Hormone
Human Serum Amyloid A (SAA)
Troponin I (TN I)
Platelet-Derived Growth Factor
Duchenne Muscular Dystrophy
SV40
Polyoma
Retroviruses
Papilloma Virus
Hepatitis B Virus

TABLE 2 - Continued

PROMOTER
Human Immunodeficiency Virus
Cytomegalovirus
Gibbon Ape Leukemia Virus

TABLE 3

Element	Inducer
MT II	Phorbol Ester (TPA) Heavy metals
MMTV (mouse mammary tumor virus)	Glucocorticoids
ß-Interferon	poly(rI)X poly(rc)
Adenovirus 5 E2	Ela
c-jun	Phorbol Ester (TPA), H_2O_2
Collagenase	Phorbol Ester (TPA)
Stromelysin	Phorbol Ester (TPA), IL-1
SV40	Phorbol Ester (TPA)
Murine MX Gene	Interferon, Newcastle Disease Virus
GRP78 Gene	A23187
α-2-Macroglobulin	IL-6
Vimentin	Serum
MHC Class I Gene H-2kB	Interferon
HSP70	Ela, SV40 Large T Antigen
Proliferin	Phorbol Ester-TPA
Tumor Necrosis Factor	FMA
Thyroid Stimulating Hormone α' Gene	Thyroid Hormone

Use of the baculovirus system will involve high level expression from the powerful polyhedron promoter.

One will typically include a polyadenylation signal to effect proper polyadenylation of the transcript. The nature of the polyadenylation signal is not believed to be crucial to the successful practice of the invention, and any such sequence may be employed. Preferred embodiments include the SV40 polyadenylation signal and the bovine growth hormone polyadenylation signal, convenient and known to function well in various target cells. Also contemplated as an element of the expression cassette is a terminator. These elements can serve to enhance message levels and to minimize read through from the cassette into other sequences.

A specific initiation signal also may be required for efficient translation of coding sequences. These signals include the ATG initiation codon and adjacent sequences. Exogenous translational control signals, including the ATG initiation codon, may need to be provided. One of ordinary skill in the art would readily be capable of determining this and providing the necessary signals. It is well known that the initiation codon must be "in-frame" with the reading frame of the desired coding sequence to ensure translation of the entire insert. The exogenous translational control signals and initiation codons can be either natural or synthetic. The efficiency of expression may be enhanced by the inclusion of appropriate transcription enhancer elements (Bittner *et al.*, 1987).

In various embodiments of the invention, the expression construct may comprise a virus or engineered construct derived from a viral genome. The ability of certain viruses to enter cells via receptor-mediated endocytosis and to integrate into host cell genome and express viral genes stably and efficiently have made them attractive candidates for the transfer of foreign genes into mammalian cells (Ridgeway, 1988; Nicolas and Rubenstein, 1988; Baichwal and Sugden, 1986; Temin, 1986). The first viruses used as vectors were DNA viruses including the papovaviruses (simian virus 40, bovine papilloma virus, and polyoma) (Ridgeway, 1988; Baichwal and Sugden, 1986) and adenoviruses (Ridgeway, 1988; Baichwal and Sugden, 1986) and adeno-associated viruses. Retroviruses also are attractive gene transfer vehicles (Nicolas and Rubenstein, 1988; Temin, 1986) as are vaccina virus (Ridgeway, 1988) and adeno-associated virus (Ridgeway, 1988). Such vectors may be used to (i) transform cell lines *in vitro*

for the purpose of expressing proteins of interest or (ii) to transform cells *in vitro* or *in vivo* to provide therapeutic polypeptides in a gene therapy scenario.

In a preferred embodiment, the vector is HSV. Because HSV is neurotropic, it has generated considerable interest in treating nervous system disorders. Moreover, the ability of HSV to establish latent infections in non-dividing neuronal cells without integrating into the host cell chromosome or otherwise altering the host cell's metabolism, along with the existence of a promoter that is active during latency. And though much attention has focused on the neurotropic applications of HSV, this vector also can be exploited for other tissues.

Another factor that makes HSV an attractive vector is the size and organization of the genome. Because HSV is large, incorporation of multiple genes or expression cassettes is less problematic than in other smaller viral systems. In addition, the availability of different viral control sequences with varying performance (temporal, strength, *etc.*) makes it possible to control expression to a greater extent than in other systems. It also is an advantage that the virus has relatively few spliced messages, further easing genetic manipulations.

HSV also is relatively easy to manipulate and can be grown to high titers. Thus, delivery is less of a problem, both in terms of volumes needed to attain sufficient MOI and in a lessened need for repeat dosings. For a review of HSV as a gene therapy vector, see Glorioso *et al.*, 1995.

V. Methods for Screening

A. Inhibitors of U_S3 and ICP4 Anti-Apoptotic Activity

In one embodiment of the present invention, there are provided methods of screening compounds for activity against U_S3's anti-apoptotic activity. These screening methods will determine the cell pathology of target cells that express U_S3, both in the presence and absence of the test compound. In another embodiment, the present invention, provides methods of screening compounds for activity against ICP4's anti-apoptotic activity. At least three different assays may be employed, as discussed below.

First, one may look at DNA fragmentation using a separative method, *e.g.*, chromatography or electrophoresis, to size fractionate the sample. As described in greater

detail in the examples, an exemplary assay involves the isolation of DNA from cells, followed by agarose gel electrophoresis and staining with ethidium bromide. DNA fragmentation, characteristic of apoptosis, will be visualized as "ladders" containing a wide range of fragment sizes.

Second, one may employ terminal deoxynucleotidyl transferase mediated dUTP-biotin nick end labeling (TUNEL) assays to measures the integrity of DNA (Gorczyca, 1993). This assay measures the fragmentation of DNA by monitoring the incorporation of labeled UTP into broken DNA strands by the enzyme terminal transferase. The incorporation can be monitored by electroscopy or by cell sorting methodologies (*e.g.*, FACS)

Third, one may examine cells using standard light or electron microscopy to assess the presence or absence of the cytopathologies characteristic of apoptosis. Those of skill in the art, applying standard methods of microscopy, will be able to assess cytopathology.

In each of these assays, a cell will be employed as the target for induction and inhibition of apoptosis. In one embodiment, the cell will be infected with HSV that expresses its own U_S3 protein. In a second embodiment, the cell will carry the U_S3 gene linked to a viral promoter. Infection with the appropriate virus will result in stimulation of the U_S3 gene and expression of U_S3. In these first two embodiments, the infection should induce apoptosis in the cell, and the expression of U_S3 should limit this effect. In a third embodiment, the cell will contain, as part of its own genetic material, an inducible version of the U_S3 gene (*i.e.*, U_S3 linked to an inducible promoter). In this situation, it will be necessary to induce apoptosis via some other mechanism, such as hypothermia, osmotic shock or ICP4 expression, and express U_S3 by inducing the promoter. The present invention may further employ ICP4 alone or in combination with U_S3 in any of the embodiments described above.

The cell is contacted with a candidate inhibitor substance in order to assess its effect on U_S3 activity. The substance may be contacted with the cell prior to, at the same time, or after the provision of U_S3. In some cases, the candidate inhibitor substance may be contacted with the cell directly. In other situations, depending on the nature and putative mechanism of action, the candidate inhibitor substance may be reformulated to provide improved uptake. For example, where antisense oligonucleotides are provided, these may advantageously be formulated in liposomes or as virally-encapsulated expression vehicles. Where polypeptides

are to be tested, it may be advantageous to provide expression vectors encoding these molecules rather than the polypeptides themselves. Essentially, the most reasonable mechanism for delivering an effective amount of the candidate inhibitor substance to the proper intracellular site will be chosen. "Effective amount," for the purposes of the screening assay, is intended to mean an amount that will cause a detectable difference, and preferably a significant difference, in the cytopathology of the cell as compared to a similar treatment of the cell without the candidate inhibitor substance. A similar protocol may be used to test the effect of a candidate substance on ICP4 activity.

Once the candidate inhibitor substance has been provided to a cell that expresses the relevant peptide(s) (U_S3, ICP4 or both), the evaluation of cytopathology may be undertaken. Depending on the type of assay used to examine cytopathology, it is possible to automate this process and test hundreds of candidates at the same time. For example, 96-well trays may be employed in which several wells are reserved for controls while the remainder comprise test substances, usually with each substance being tested at several different amounts.

B. Screening for Modulators of US3 function

In certain embodiments, the present invention concerns a method for identifying modulators of U_S3 function. Such modulators may be inhibitors or stimulators of such a function. It is contemplated that this screening technique will prove useful in the general identification of any compound that will serve the purpose of inhibiting or stimulating U_S3-mediated kinase activity.

The active compounds may include fragments or parts of naturally-occurring compounds or may be only found as active combinations of known compounds which are otherwise inactive. However, prior to testing of such compounds in humans or animal models, it will possibly be necessary to test a variety of candidates to determine which have potential.

Accordingly, in screening assays to identify pharmaceutical agents which modulate U_S3 kinase activity, it is proposed that compounds isolated from natural sources, such as animals, bacteria, fungi, plant sources, including leaves and bark, and marine samples may be assayed as candidates for the presence of potentially useful pharmaceutical agents. It will be understood

that the pharmaceutical agents to be screened could also be derived or synthesized from chemical compositions or man-made compounds.

In these embodiments, the present invention is directed to a method for determining the ability of a candidate substance to inhibit or stimulate a protein kinase assay, the method including generally the steps of:

(a) obtaining an enzyme composition comprising a protein kinase, preferably U_S3 protein kinase that is capable of phosphorylating threonine/serine residue;

(b) admixing a candidate substance with the enzyme composition; and

(c) determining the ability of the candidate substance to inhibit or stimulate threonine/serine phosphorylation

To identify a candidate substance as being capable of modulating protein phosphorylation, one would first obtain an enzyme composition that is capable of phosphorylating threonine/serine residues on a protein of interest. Naturally, one would measure or determine the phosphorylation activity of the threonine/serine kinase composition in the absence of the added candidate substance. One would then add the candidate substance to the threonine/serine kinase composition and re-determine the ability of the threonine/serine kinase composition to phosphorylate threonine/serine residues on a test protein in the presence of the candidate substance. A candidate substance which reduces the phosphorylation activity of the threonine/serine kinase composition relative to the activity in its absence is indicative of a candidate substance with inhibitor capability. Likewise, a candidate substance which increases the phosphorylation activity of the threonine/serine kinase composition relative to the activity in its absence is indicative of a candidate substance with stimulatory capability

The candidate screening assay is quite simple to set up and perform, after obtaining a relatively purified preparation of the enzyme, either from native or recombinant sources, one will admix a candidate substance with the enzyme preparation, under conditions which would allow the enzyme to perform its threonine/serine phosphorylation function but for inclusion of a candidate substance. In this fashion, one can measure the ability of the candidate substance to reduce or increase the threonine/serine phosphorylation activity relatively in the presence of the candidate substance.

"Effective amounts" in certain circumstances are those amounts effective to reproducibly reduce or increase threonine/serine kinase activity, or to inhibit or induce the apoptosis of cells, in comparison to their normal levels. Compounds that achieve significant appropriate changes in activity will be used. If desired, a battery of compounds may be screened *in vitro* to identify agents for use in the present invention.

Significant decrease in threonine/serine phosphorylation, *e.g.*, as measured using immunoblotting techniques with anti-phosphorylation antibodies, are represented by a reduction in protein phosphorylation levels of at least about 30%-40%, and most preferably, by decreases of at least about 50%, with higher values of course being possible. Threonine/serine kinase assays that measure threonine/serine phosphorylation are well known in the art and may be conducted *in vitro* or *in vivo*. Likewise increases in threonine/serine phosphorylation are represented by an increase in protein phosphorylation levels of at least about 30%-40%, and most preferably, by increases of at least about 50%, with higher values of course being possible.

Assays for apoptosis will measure certain cellular events, including nuclear condensation, DNA fragmentation, cytoplasmic membrane blebbing and, ultimately, irreversible cell death. The methodology for such measurements is well known to those of skill in the art. A significant alteration in apoptosis is represented by an increase or decrease of at least about 30%-40% as compared to normal, and most preferably, of at least about 50%, with more significant increases or decreases also being possible. Therefore, if a candidate substance exhibited inhibition or induction of apoptosis in this type of study, it would likely be a suitable compound for use in the present invention.

Quantitative *in vitro* testing is not a requirement of the invention as it is generally envisioned that the candidate substance will often be selected on the basis of their known properties or by structural and/or functional comparison to those agents already known to have an effect on threonine/serine kinases.

It will, of course, be understood that all the screening methods of the present invention are useful in themselves notwithstanding the fact that effective candidates may not be found. The invention provides methods for screening for such candidates, not solely methods of finding them.

VI. Methods for the Inhibition of Apoptosis

In one embodiment of the present invention, there are provided methods for the inhibition of apoptosis in a cell. This is particularly useful where one seeks to immortalize a cell or, at a minimum, increase the longevity of a cell. This permits one to maintain that cell in culture for extended periods of time, perhaps indefinitely. Immortalized cells are useful primarily as factories for production of viral vectors or proteins of interest, but it also may be important to immortalize cell simply so that they may be studied *in vitro* with greater ease. In addition, though many viruses provide promise as gene therapeutic vectors, these vectors may trigger apoptosis in the cells they infect. Blocking virally-induced apoptosis will prevent cell death caused by these therapeutic vectors. As mentioned above, adenovirus, papilloma viruses, retrovirus, adeno-associated virus and HSV, for example, are candidate gene therapeutic vectors that could benefit from this application.

The general approach to inhibiting apoptosis, according to the present invention, will be to provide a cell with an U_S3 polypeptide, an ICP4 polypeptide or both, thereby permitting the inhibitory activity of U_S3, ICP4 or both to take effect. While it is conceivable that the protein may be delivered directly, a preferred embodiment involves providing a nucleic acid encoding the polypeptide, *i.e.*, an U_S3 gene, an $\alpha 4$ gene or both, to the cell. Following this provision, the polypeptide is synthesized by the host cell's transcriptional and translational machinery, as well as any that may be provided by the expression construct. Cis-acting regulatory elements necessary to support the expression of the U_S3 or $\alpha 4$ gene will be provided, as described above, in the form of an expression construct. It also is possible that, in the case of an HSV-infected cell, expression of the virally-encoded U_S3 or ICP4 could be stimulated or enhanced, or the expressed polypeptide stabilized, thereby achieving the same or similar effect.

In order to effect expression of constructs encoding U_S3 and/or ICP genes, the expression construct must be delivered into a cell. As described above in the discussion of viral vectors, one mechanism for delivery is via viral infection, where the expression construct is encapsidated in a viral particle which will deliver either a replicating or non-replicating nucleic acid. The preferred embodiment is an HSV vector, although virtually any vector would suffice. Similarly, where viral vectors are used to delivery other therapeutic genes, inclusion in these

vectors of an U_S3 and/or $\alpha 4$ gene advantageously will protect the cell from virally induced apoptosis.

Several non-viral methods for the transfer of expression constructs into cultured mammalian cells also are contemplated by the present invention. These include calcium phosphate precipitation (Graham and Van Der Eb, 1973; Chen and Okayama, 1987; Rippe *et al.*, 1990) DEAE-dextran (Gopal, 1985), electroporation (Tur-Kaspa *et al.*, 1986; Potter *et al.*, 1984), direct microinjection (Harland and Weintraub, 1985), DNA-loaded liposomes (Nicolau and Sene, 1982; Fraley *et al.*, 1979) and lipofectamine-DNA complexes, cell sonication (Fechheimer *et al.*, 1987), gene bombardment using high velocity microprojectiles (Yang *et al.*, 1990), and receptor-mediated transfection (Wu and Wu, 1987; Wu and Wu, 1988). Some of these techniques may be successfully adapted for *in vivo* or *ex vivo* use, as discussed below.

In another embodiment of the invention, the expression construct may simply consist of naked recombinant DNA or plasmids. Transfer of the construct may be performed by any of the methods mentioned above which physically or chemically permeabilize the cell membrane. This is particularly applicable for transfer *in vitro*, but it may be applied to *in vivo* use as well. Another embodiment of the invention for transferring a naked DNA expression construct into cells may involve particle bombardment. This method depends on the ability to accelerate DNA coated microprojectiles to a high velocity allowing them to pierce cell membranes and enter cells without killing them (Klein *et al.*, 1987). Several devices for accelerating small particles have been developed. One such device relies on a high voltage discharge to generate an electrical current, which in turn provides the motive force (Yang *et al.*, 1990). The microprojectiles used have consisted of biologically inert substances such as tungsten or gold beads.

In a further embodiment of the invention, the expression construct may be entrapped in a liposome. Liposomes are vesicular structures characterized by a phospholipid bilayer membrane and an inner aqueous medium. Multilamellar liposomes have multiple lipid layers separated by aqueous medium. They form spontaneously when phospholipids are suspended in an excess of aqueous solution. The lipid components undergo self-rearrangement before the formation of closed structures and entrap water and dissolved solutes between the lipid bilayers (Ghosh and Bachhawat, 1991). Also contemplated are lipofectamine-DNA complexes.

Liposome-mediated nucleic acid delivery and expression of foreign DNA in vitro has been very successful. Wong *et al.* (1980) demonstrated the feasibility of liposome-mediated delivery and expression of foreign DNA in cultured chick embryo, HeLa and hepatoma cells. In certain embodiments of the invention, the liposome may be complexed with a hemagglutinating virus (HVJ). This has been shown to facilitate fusion with the cell membrane and promote cell entry of liposome-encapsulated DNA (Kaneda *et al.*, 1989). In other embodiments, the liposome may be complexed or employed in conjunction with nuclear non-histone chromosomal proteins (HMG-1) (Kato *et al.*, 1991). In yet further embodiments, the liposome may be complexed or employed in conjunction with both HVJ and HMG-1. In other embodiments, the delivery vehicle may comprise a ligand and a liposome. Where a bacterial promoter is employed in the DNA construct, it also will be desirable to include within the liposome an appropriate bacterial polymerase.

Other expression constructs which can be employed to deliver a nucleic acid encoding an U_S3 and/or $\alpha 4$ transgene into cells are receptor-mediated delivery vehicles. These take advantage of the selective uptake of macromolecules by receptor-mediated endocytosis in almost all eukaryotic cells. Because of the cell type-specific distribution of various receptors, the delivery can be highly specific (Wu and Wu, 1993).

Receptor-mediated gene targeting vehicles generally consist of two components: a cell receptor-specific ligand and a DNA-binding agent. Several ligands have been used for receptor-mediated gene transfer. The most extensively characterized ligands are asialoorosomucoid (ASOR) (Wu and Wu, 1987) and transferrin (Wagner *et al.*, 1990). Recently, a synthetic neoglycoprotein, which recognizes the same receptor as ASOR, has been used as a gene delivery vehicle (Ferkol *et al.*, 1993; Perales *et al.*, 1994). Mannose can be used to target the mannose receptor on liver cells. Also, antibodies to CD5 (CLL), CD22 (lymphoma), CD25 (T-cell leukemia) and MAA (melanoma) can similarly be used as targeting moieties. In other embodiments, the delivery vehicle may comprise a ligand and a liposome.

Primary mammalian cell cultures may be prepared in various ways. In order for the cells to be kept viable while *in vitro* and in contact with the expression construct, it is necessary to ensure that the cells maintain contact with the correct ratio of oxygen and carbon dioxide and

nutrients but are protected from microbial contamination. Cell culture techniques are well documented and are disclosed herein by reference (Freshner, 1992).

One embodiment of the foregoing involves the use of gene transfer to immortalize cells for the production of proteins. The gene for the protein of interest may be transferred as described above into appropriate host cells followed by culture of cells under the appropriate conditions. The gene for virtually any polypeptide may be employed in this manner. The generation of recombinant expression vectors, and the elements included therein, are discussed above. Alternatively, the protein to be produced may be an endogenous protein normally synthesized by the cell in question.

Examples of useful mammalian host cell lines are Vero and HeLa cells and cell lines of Chinese hamster ovary, W138, BHK, COS-7, 293, HepG2, 3T3, RIN and MDCK cells. In addition, a host cell strain may be chosen that modulates the expression of the inserted sequences, or modifies and process the gene product in the manner desired. Such modifications (*e.g.*, glycosylation) and processing (*e.g.*, cleavage) of protein products may be important for the function of the protein. Different host cells have characteristic and specific mechanisms for the post-translational processing and modification of proteins. Appropriate cell lines or host systems can be chosen to insure the correct modification and processing of the foreign protein expressed.

A number of selection systems may be used including, but not limited to, HSV thymidine kinase, hypoxanthine-guanine phosphoribosyltransferase and adenine phosphoribosyltransferase genes, in *tk-*, *hgprt-* or *aprt-* cells, respectively. Also, anti-metabolite resistance can be used as the basis of selection for *dhfr*, that confers resistance to; *gpt*, that confers resistance to mycophenolic acid; *neo*, that confers resistance to the aminoglycoside G418; and *hygro*, that confers resistance to hygromycin.

Animal cells can be propagated *in vitro* in two modes: as non-anchorage dependent cells growing in suspension throughout the bulk of the culture or as anchorage-dependent cells requiring attachment to a solid substrate for their propagation (*i.e.*, a monolayer type of cell growth).

Non-anchorage dependent or suspension cultures from continuous established cell lines are the most widely used means of large scale production of cells and cell products. However, suspension cultured cells have limitations, such as tumorigenic potential and lower protein production than adherent cells.

Large scale suspension culture of mammalian cells in stirred tanks is a common method for production of recombinant proteins. Two suspension culture reactor designs are in wide use - the stirred reactor and the airlift reactor. The stirred design has successfully been used on an 8000 liter capacity for the production of interferon (Phillips *et al.*, 1985; Mizrahi, 1983). Cells are grown in a stainless steel tank with a height-to-diameter ratio of 1:1 to 3:1. The culture is usually mixed with one or more agitators, based on bladed disks or marine propeller patterns. Agitator systems offering less shear forces than blades have been described. Agitation may be driven either directly or indirectly by magnetically coupled drives. Indirect drives reduce the risk of microbial contamination through seals on stirrer shafts.

The airlift reactor, also initially described for microbial fermentation and later adapted for mammalian culture, relies on a gas stream to both mix and oxygenate the culture. The gas stream enters a riser section of the reactor and drives circulation. Gas disengages at the culture surface, causing denser liquid free of gas bubbles to travel downward in the downcomer section of the reactor. The main advantage of this design is the simplicity and lack of need for mechanical mixing. Typically, the height-to-diameter ratio is 10:1. The airlift reactor scales up relatively easily, has good mass transfer of gases and generates relatively low shear forces.

VII. Methods for the Induction of Apoptosis

In another embodiment of the present invention, there is contemplated the method of inducing apoptosis in HSV-infected cells, *i.e.*, blocking the apoptotic function of U_S3, ICP4 or both. In this way, it may be possible to curtail viral infection by bringing about a premature death of the infected cell. In addition, it may prove effective to use this sort of therapeutic intervention in combination with more traditional chemotherapies, such as the administration acyclovir.

The general form that this aspect of the invention will take is the provision, to a cell, of an agent that will inhibit U_S3 function, ICP4 function or indeed inhibit the function of both. Four such agents are contemplated as outlined below.

First, one may employ an antisense nucleic acid that will hybridize either to the U_S3 gene or the U_S3 transcript, thereby preventing transcription or translation, respectively. Likewise, one may employ an antisense nucleic acid that will hybridize to an α4 gene or an α4 transcript. The considerations relevant to the design of antisense constructs have been presented above.

Second, one may utilize an U_S3-binding protein or peptide, for example, a peptidomimetic or an antibody that binds immunologically to an U_S3, the binding of either will block or reduce the activity of an U_S3. Again the same is true of ICP4 in that one may utilize an ICP4-binding protein or peptide, for example, a peptidomimetic or an antibody that binds immunologically to an ICP4, the binding of either resulting in a block or reduction of ICP4 activity. The methods of making and selecting peptide binding partners and antibodies are well known to those of skill in the art.

Third, one may provide to the cell an antagonist of U_S3 for example, the in the case of U_S3 phosphorylation target sequence, alone or coupled to another agent. Equally one may provide to the cell an antagonist of ICP4, for example, the transactivation target sequence, alone or coupled to another agent. And fourth, one may provide an agent that binds to the U_S3 or ICP4 target without the same functional result as would arise with U_S3 or ICP4 binding.

Provision of an U_S3 gene, an U_S3-binding protein, an U_S3 antagonist, or an α4 gene, an ICP4-binding protein, or an ICP4 antagonist, would be according to any appropriate pharmaceutical route. The formulation of such compositions and their delivery to tissues is discussed below. The method by which the nucleic acid, protein or chemical is transferred, along with the preferred delivery route, will be selected based on the particular site to be treated. Those of skill in the art are capable of determining the most appropriate methods based on the relevant clinical considerations.

Many of the gene transfer techniques that generally are applied *in vitro* can be adapted for *ex vivo* or *in vivo* use. For example, selected organs including the liver, skin, and muscle

tissue of rats and mice have been bombarded *in vivo* (Yang *et al.*, 1990; Zelenin *et al.*, 1991). Naked DNA also has been used in clinical settings to effect gene therapy. These approaches may require surgical exposure of the tumor tissue or direct intratumoral injection. Nicolau *et al.* (1987) accomplished successful liposome-mediated gene transfer in rats after intravenous injection.

Dubensky *et al.* (1984) successfully injected polyomavirus DNA in the form of $CaPO_4$ precipitates into liver and spleen of adult and newborn mice demonstrating active viral replication and acute infection. Benvenisty and Neshif (1986) also demonstrated that direct intraperitoneal injection of $CaPO_4$ precipitated plasmids results in expression of the transfected genes. Thus, it is envisioned that DNA encoding an antisense construct also may be transferred in a similar manner *in vivo*.

Where the embodiment involves the use of an antibody that recognizes an U_S3 or an ICP4 polypeptide, consideration must be given to the mechanism by which the antibody is introduced into the cell cytoplasm. This can be accomplished, for example, by providing an expression construct that encodes a single-chain antibody version of the antibody to be provided. Most of the discussion above relating to expression constructs for antisense versions of the respective genes will be relevant to this aspect of the invention. Alternatively, it is possible to present a bifunctional antibody, where one antigen binding arm of the antibody recognizes an U_S3 or ICP4 polypeptide and the other antigen binding arm recognizes a receptor on the surface of the cell to be targeted. Examples of suitable receptors would be an HSV glycoprotein such as gB, gC, gD, or gH. In addition, it may be possible to exploit the Fc-binding function associated with HSV gE, thereby obviating the need to sacrifice one arm of the antibody for purposes of cell targeting.

Advantageously, one may combine this approach with more conventional chemotherapeutic options. Acyclovir is an active agent against HSV-1 and HSV-2. The drug inhibits actively replicating herpes virus but is not active against latent virus. Acyclovir is available in three formulations. For topical use, a five percent ointment produces therapeutic drug levels in mucocutaneous lesions. For systemic use, acyclovir may be administered orally or intravenously. The usual intravenous dosage in adults with normal renal function is 5 mg/kg infused at a constant rate over one hour and given every eight hours; this dosage produces peak

plasma levels at about 10 g/ml. For HSV encephalitis, twice this dose is used. The usual adult oral dosage is 200 mg, five times daily, which produces plasma levels that are less than 10% as high as those achieved with intravenous administration; even these levels are inhibitory to the virus, however. Acyclovir is given in an oral dosage of 800 mg five times daily for the treatment of herpes zoster, although oral administration generally is reserved for patients with severe symptoms. A three percent opthalmic preparation produces inhibitory drug levels in the aqueous humor and is effective for herpes keratitis.

In contemplating combinatorial aspects of the present invention, it will be possible to combine the U_S3 therapeutic compositions of the present invention with other apoptosis inducing or inhibiting compositions. For example the present inventors have show that it is possible to affect apoptosis using compositions derived from ICP4 protein or polypeptide (SEQ ID NO:2) and the $\alpha 4$ gene or gene constructs protein or polypeptide (SEQ ID NO:1). Thus in light of the teachings presented herein, it will be possible for one of ordinary skill in the art to combine the U_S3 therapeutic compositions with those derived from ICP4 and $\alpha 4$ to affect apoptosis as described herein below.

To kill cells, inhibit cell growth, or induce apoptosis as defined above, using the methods and compositions of the present invention, one would generally contact a "target" cell with a U_S3 and/or an ICP4 expression construct alone or in combination with at least one other agent. These compositions would be provided in a combined amount effective to kill or induce apoptosis of the cell. This process may involve contacting the cells with the expression construct and the agent(s) or factor(s) at the same time. This may be achieved by contacting the cell with a single composition or pharmacological formulation that includes both agents, or by contacting the cell with two distinct compositions or formulations, at the same time, wherein one composition includes the expression construct and the other includes the agent.

Alternatively, the gene therapy treatment may precede or follow the other agent treatment by intervals ranging from minutes to weeks. In embodiments where the other agent and expression construct are applied separately to the cell, one would generally ensure that a significant period of time did not expire between the time of each delivery, such that the agent and expression construct would still be able to exert an advantageously combined effect on the cell. In such instances, it is contemplated that one would contact the cell with both modalities

within about 12-24 hours of each other and, more preferably, within about 6-12 hours of each other, with a delay time of only about 12 hours being most preferred. In some situations, it may be desirable to extend the time period for treatment significantly, however, where several days (2, 3, 4, 5, 6 or 7) to several weeks (1, 2, 3, 4, 5, 6, 7 or 8) lapse between the respective administrations.

It also is conceivable that more than one administration of either U_S3 or the other agent will be desired. Various combinations may be employed, where U_S3 is "A" and the other agent is "B", as exemplified below:

A/B/A B/A/B B/B/A A/A/B B/A/A A/B/B B/B/B/A B/B/A/B

A/A/B/B A/B/A/B A/B/B/A B/B/A/A B/A/B/A B/A/A/B B/B/B/A

A/A/A/B B/A/A/A A/B/A/A A/A/B/A A/B/B/B B/A/B/B B/B/A/B

Other combinations are contemplated where ICP4 and α4 may also be used. Again, to achieve apoptosis, both agents are delivered to a cell in a combined amount effective induce cell death.

The skilled artisan is directed to "Remington's Pharmaceutical Sciences" 15th Edition, chapter 33, in particular pages 624-652. Some variation in dosage will necessarily occur depending on the condition of the subject being treated. The person responsible for administration will, in any event, determine the appropriate dose for the individual subject. Moreover, for human administration, preparations should meet sterility, pyrogenicity, general safety and purity standards as required by FDA Office of Biologics standards.

The inventors propose that the regional delivery of U_S3 expression constructs and/or ICP4 expression constructs to patients with HSV linked disease will be a very efficient method for delivering a therapeutically effective gene to counteract the clinical disease. Similarly, the chemotherapy may be directed to a particular, affected region of the subjects body. Alternatively, systemic delivery of expression construct and/or the agent may be appropriate in certain circumstances, for example, where extensive metastasis has occurred.

In addition to combining U_S3-targeted therapies with chemotherapies, it also is contemplated that combination with other gene therapies will be advantageous. For example,

targeting of U_S3 and α4 mutations at the same time may produce an improved apoptotic treatment.

It also should be pointed out that any of the foregoing therapies may prove useful by themselves in treating a U_S3 related disease. In this regard, reference to chemotherapeutics and non-U_S3 gene therapy in combination should also be read as a contemplation that these approaches may be employed separately.

VIII. Methods for the Inhibition of Virus-Induced Cell Death *In Vivo*

In another embodiment of the present invention, there are provided methods for the inhibition of cell death induced *in vivo* by any cause comprising the provision of U_S3 polypeptides or U_S3 genes, or ICP4 polypeptides and α4 genes or combinations of the two. Also contemplated in this aspect of the invention is the stimulation of viral U_S3 expression, and/or stimulation of viral ICP4 expression or stabilization of the virally-expressed U_S3 polypeptide and or ICP4 polypeptide. Though inhibition of apoptosis generally is thought of as advantageous to the virus, it may be desirable to effect this result as part of a method of treating a viral infection. For example, if the host cell remains viable, the virus may continue to replicate; alternatively, if apoptosis were occurring, the virus might be inclined to "go latent" in the neural ganglia, where chemotherapeutic intervention is not helpful. Thus, by preventing early death of the cell, U_S3 and/or ICP4 may cause the virus to remain susceptible to treatment where it otherwise would escape.

The mechanisms for delivering proteins and nucleic acids to a cell are discussed elsewhere in this document and need not be repeated here. The use of standard chemotherapeutics has been presented in the preceding section, and is incorporated in this section.

IX. Pharmaceuticals and *In Vivo* Methods for the Treatment of Disease

Aqueous pharmaceutical compositions of the present invention will have an effective amount of an U_S3 and/or α4 expression construct, an antisense U_S3 and/or α4 expression construct, an expression construct that encodes a therapeutic gene along with U_S3 and/or α4, a protein that inhibits U_S3 and/or α4 function, such as an anti-U_S3 antibody or an anti-ICP4

antibody, respectively, or an U_S3 polypeptide and/or an ICP4 polypeptide. Such compositions generally will be dissolved or dispersed in a pharmaceutically acceptable carrier or aqueous medium. An "effective amount," for the purposes of therapy, is defined at that amount that causes a clinically measurable difference in the condition of the subject. This amount will vary depending on the substance, the condition of the patient, the type of treatment, the location of the lesion, *etc.*

The phrases "pharmaceutically or pharmacologically acceptable" refer to molecular entities and compositions that do not produce an adverse, allergic or other untoward reaction when administered to an animal, or human, as appropriate. As used herein, "pharmaceutically acceptable carrier" includes any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents and the like. The use of such media and agents for pharmaceutical active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the active ingredients, its use in the therapeutic compositions is contemplated. Supplementary active ingredients, such as other anti-cancer agents, can also be incorporated into the compositions.

In addition to the compounds formulated for parenteral administration, such as those for intravenous or intramuscular injection, other pharmaceutically acceptable forms include, *e.g.*, tablets or other solids for oral administration; time release capsules; and any other form currently used, including cremes, lotions, mouthwashes, inhalants and the like.

The active compounds of the present invention will often be formulated for parenteral administration, *e.g.*, formulated for injection *via* the intravenous, intramuscular, subcutaneous, or even intraperitoneal routes. The preparation of an aqueous composition that contains glycosylceramide synthesis inhibitory compounds alone or in combination with a chemotherapeutic agent as active ingredients will be known to those of skill in the art in light of the present disclosure. Typically, such compositions can be prepared as injectables, either as liquid solutions or suspensions; solid forms suitable for using to prepare solutions or suspensions upon the addition of a liquid prior to injection can also be prepared; and the preparations can also be emulsified.

Solutions of the active compounds as free base or pharmacologically acceptable salts can be prepared in water suitably mixed with a surfactant, such as hydroxypropylcellulose.

Dispersions can also be prepared in glycerol, liquid polyethylene glycols, and mixtures thereof and in oils. Under ordinary conditions of storage and use, these preparations contain a preservative to prevent the growth of microorganisms.

The pharmaceutical forms suitable for injectable use include sterile aqueous solutions or dispersions; formulations including sesame oil, peanut oil or aqueous propylene glycol; and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersions. In many cases, the form must be sterile and must be fluid to the extent that easy syringability exists. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms, such as bacteria and fungi.

The active compounds may be formulated into a composition in a neutral or salt form. Pharmaceutically acceptable salts, include the acid addition salts (formed with the free amino groups of the protein) and which are formed with inorganic acids such as, for example, hydrochloric or phosphoric acids, or such organic acids as acetic, oxalic, tartaric, mandelic, and the like. Salts formed with the free carboxyl groups can also be derived from inorganic bases such as, for example, sodium, potassium, ammonium, calcium, or ferric hydroxides, and such organic bases as isopropylamine, trimethylamine, histidine, procaine and the like.

The carrier also can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyethylene glycol, and the like), suitable mixtures thereof, and vegetable oils. The proper fluidity can be maintained, for example, by the use of a coating, such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. The prevention of the action of microorganisms can be brought about by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, thimerosal, and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars or sodium chloride. Prolonged absorption of the injectable compositions can be brought about by the use in the compositions of agents delaying absorption, for example, aluminum monostearate and gelatin.

Sterile injectable solutions are prepared by incorporating the active compounds in the required amount in the appropriate solvent with various of the other ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the various sterilized active ingredients into a sterile vehicle which contains the

basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum-drying and freeze-drying techniques which yield a powder of the active ingredient plus any additional desired ingredient from a previously sterile-filtered solution thereof.

In certain cases, the therapeutic formulations of the invention could also be prepared in forms suitable for topical administration, such as in cremes and lotions. These forms may be used for treating skin-associated diseases, such as various sarcomas. In certain other cases, the formulation will be geared for administration to the central nervous system, *e.g.*, the brain.

Upon formulation, solutions will be administered in a manner compatible with the dosage formulation and in such amount as is therapeutically effective. The formulations are easily administered in a variety of dosage forms, such as the type of injectable solutions described above, with even drug release capsules and the like being employable.

For parenteral administration in an aqueous solution, for example, the solution should be suitably buffered if necessary and the liquid diluent first rendered isotonic with sufficient saline or glucose. These particular aqueous solutions are especially suitable for intravenous, intramuscular, subcutaneous and intraperitoneal administration. In this connection, sterile aqueous media which can be employed will be known to those of skill in the art in light of the present disclosure. For example, one dosage could be dissolved in 1 mL of isotonic NaCl solution and either added to 1000 mL of hypodermoclysis fluid or injected at the proposed site of infusion, (see for example, "Remington's Pharmaceutical Sciences" 15th Edition, pages 1035-1038 and 1570-1580). Some variation in dosage will necessarily occur depending on the condition of the subject being treated. The person responsible for administration will, in any event, determine the appropriate dose for the individual subject.

X. Examples

The following examples are included to demonstrate preferred embodiments of the present invention. It should be appreciated by those of skill in the art that that techniques disclosed in the examples which follow represent techniques discovered by the inventors to function well in the practice of the invention, and thus can be considered to constitute preferred

modes for its practice. However, those of skill in the art should, in light of the present disclosure, appreciate that many changes can be made in the specific embodiments which are disclosed and still obtain a like or similar results without departing from the spirit and scope of the invention.

EXAMPLE 1:

Materials And Methods

Cells and Viruses. Vero and BHK cells were originally obtained from ATCC. HSV-1(F) is the prototype HSV-1 strain used in this laboratory (Ejercito *et al.*, 1967). In recombinant R325 derived from HSV-1(F), approximately 800 bp comprising the carboxyl-terminal half of the α4 gene and grows only in a Vero cell line expressing the α4 gene (DeLuca *et al.*, 1985). Both the virus and the cell line were kind gifts of Neal DeLuca (University of Pittsburgh). The U_S3 gene was deleted from the recombinant R7041 (Longnecker *et al.*, 1987) and then repaired to yield R7306).

Plasmids and Cosmids. The plasmid pRB5166 was constructed by cloning into the SalI/BamHI sites of the pACYC184 vector a HSV-1 DNA fragment that extends from the Sal I site at + 177 with respect to the α4 gene transcription start site and includes the entire BamHI P and BamHI S fragments. The cosmid cloning vector pRB78 was constructed as follows. The multiple cloning site of the Stratagene Supercosl (La Jolla CA, cat#251301) was cleaved with EcoRI and replaced with an oligonucleotides containing EcoRI/PacI/Sse8387I/SpeI/BamHI/NdeI/EcoRV/PacI/EcoRI cloning sites. The PacI restriction site, absent in HSV-1, serves to liberates the cloned HSV-1(F) DNA fragments from the vector. HSV-1(F) viral DNA was prepared from virions as previously described. To construct cosmid pBC1004, which contains the HSV-1(F) sequence n133052 through n17059, viral DNA was partially digested with Sau3A I, dephosphorylated and ligated into the BamHI site of the pRB78 cosmid vector previously linearized with Xba I. The DNA was then packaged using Stratagene Gigapack XLII (La Jolla, CA), following the manufacturer's instructions. E. coli L-1 Blue MR was then infected and ampicillin-resistant colonies were screened by restriction enzyme analysis. Insert termini were sequenced to verify mapping. Cosmid pBC1008 was constructed by cloning the *Bgl*II F-H fragment (HSV-1(F) n106750 through n142759) into the

*Bam*HI site of pRB78 vector. Cosmid pBC1009 was constructed as follows. An *Nsi*I/*Sca*I DNA fragment was isolated from a double digest of viral DNA. The fragment (HSV-1(F) n137538 through n18545) was ligated into the Sse8387I/*Eco*RV sites of pBR78. Cosmids PBC1008 and PBC1009 were mapped by restriction enzyme analysis and insert termini were sequenced (FIG. 1).

Electron photomicroscopy. Vero cells infected with HSV-1(F) or d120 were incubated for 20 hrs at 37°C. Cells were fixed in 2% glutaraldehyde in PBS for 60 min at 4°C, post-fixed with 1% osmium tetroxide, en bloc stained with uranyl acetate (5mg/ml), dehydrated in acetone, and embedded in Epon 812. Thin sections were examined either unstained or poststained with uranyl acetate and lead hydroxide. The cells were photographed at 6000 X in a Siemens 101 electron microscope.

Light photomicroscopy. Cells were labeled with biotinylated dUTP. At indicated times the cells were fixed in ice-cold methanol at -20°C and air-dried, then rinsed in phosphate buffered saline, reacted for 15 min at room temperature with 40 ml of a solution containing 1x terminal transferase buffer (Promega), 1 mM $CoCl_2$, 0.05 mg/ml bovine serum albumin (BSA), 0.5 nmoles biotin-16-dUTP (Boehringer Mannheim Biochemicals) and 3 units of terminal transferase (TdT, Promega), rinsed extensively in PBS, reacted for 30 min at room temperature with 40 ml of a solution containing Texas red-conjugated avidin in 4x SSC (1x SSC is 0.15 M NaCl, 0.015 M Na-citrate), 0.1 % Triton X-100, 5% (w/v) nonfat dry milk, extensively rinsed with PBS, mounted in 10% PBS in glycerol and examined under a Zeiss confocal fluorescence microscope. The images were captured under identical settings with the software provided by Zeiss and printed in a Tektronix 440 phaser printer. Cells were mock infected, 37°C, 20 hrs; infected with d120 virus, 37°C 20 hrs; infected with HSV-1(F), 37°C, 20 hrs; infected with d120 virus, 37°C 30 hrs; mock-infected, 39.5°C, 30 hrs.; infected with d120 virus, 39.5°C, 30 hrs.; and infected with HSV-1(F), 39.5° C, 30 hrs.

DNA Fragmentation Assay. Vero cells or E5 cells were infected with HSV-1(F), d120 mutant, or HSV-1 tsHA1 mutant and maintained at 37°C or 39.5°C in the absence or in the presence of phosphonoacetic acid. At 30 hrs after infection, 2 x 10^6 cells per sample were collected, washed in PBS, lysed in a solution containing 10 mM Tris-HCl, pH 8.0, 10 mM EDTA, and 0.5 % Triton X-100, and centrifuged at 12,000 rpm for 25 min in an Eppendorf

microcentrifuge to pellet chromosomal DNA. Supernatant fluids were digested with 0.1 mg RNase A per ml at 37°C for 1 hr, for 2 hrs with 1 mg proteinase K per ml at 50°C in the presence of 1 % sodium dodecylsulphate (SDS), extracted with phenol and chloroform, and precipitated in cold ethanol and subjected to electrophoresis on horizontal 1.5 % agarose gels containing 5 mg of ethidium bromide per ml. DNA was visualized by UV light transillumination. Photographs were taken with the aid of a computer-assisted image processor (Eagle Eye II, Stratagene).

Construction of recombinant viruses by marker rescue. Recombinant viruses were constructed using a modification of the technique originally described by Post and Roizman (1981). Vero cells were transfected with plasmid or cosmid DNA using Lipofectamin, according to the manufacturer's instructions. At 6 hr after transfection, the cells were exposed to 0.1 to 1 PFU of HSV-1(Kos)d120 per cell. Recombinant viruses were isolated from single plaques and grown in Vero cells. To obtain HSV-1 120KR, Vero cells were exposed to 10 PFU of HSV-1(KOS)d120 per cell. The infected cells were harvested at 48 hr post infection, frozen-thawn and sonicated, and then serially diluted and titered on Vero cells. Recombinant viruses were isolated from single plaques and grown in Vero cells.

EXAMPLE 2

An HSV-1 mutant deleted in ICP4 induces apoptosis.

In this series of studies, Vero cells infected with wild-type or the d120 mutant were examined for morphologic evidence of apoptosis. Vero cells were fixed and harvested at 20 to 24 hrs after infection with wild-type or d120, embedded, sectioned, and examined in a Siemens 101 electron microscope. The cells infected with wild-type virus showed typical infected cell morphology, *i.e.*, marginated chromatin, separation of inner and outer nuclear membranes, and accumulation of virus particles in some but not all cells. Cells infected with the d120 deletion mutant exhibited extensive condensation of chromatin, obliteration of the nuclear membrane, and extensive vacuolization and blebbing of the cytoplasm. It was estimated that approximately 40 to 50% of the infected cells exhibited some or all of the morphologic changes described above.

In another series of studies, Vero cells were mock-infected or infected with 10 PFU of either the wild-type or the d120 mutant virus per cell. After 20 hrs of incubation at 37° C the

cells were fixed, labeled with biotinylated dUTP in the presence of terminal transferase, and then reacted with fluorescent avidin. Mock-infected cells or cells infected with wild-type virus showed no sign of labeling with biotinylated dUTP by terminal transferase, whereas cells infected with d120 and maintained at the same temperature showed extensive fluorescence due to the reaction of fluorescent avidin to biotinylated dUTP incorporated at the DNA termini created by the cleavage of DNA.

In the third series of studies, replicate Vero cell cultures were infected with 10 PFU of either HSV-1(F) or d120 per cell and incubated at 37°C. The study also included a Vero cell culture infected with HSV-1(F), overlaid with medium containing 300 μg of phosphonoacetate per ml and incubated at 37°C, and a set of Vero cell cultures infected with 10 PFU of HSV-1 tsHA1 and incubated at either 37°C or 39.5°C. This concentration of phosphonoacetate completely inhibits viral DNA synthesis and blocks the expression of γ_2 genes dependent on viral synthesis for their expression. The cells were harvested at 30 hrs after infection, lysed, and centrifuged to pellet the chromosomal DNA. The supernatant fluids were processed as described above and subjected to electrophoresis in agarose gels to test for the presence of soluble, fragmented DNA.

The results were as follows. Cells infected with d120 deletion mutant yielded high amounts of fragmented DNA which were readily visible on agarose gels stained with ethidium bromide. These ladders were not seen in agarose gels containing electrophoretically separated extracts of wild-type infected cells or E5 cells infected with d120. When Vero cells were incubated in medium containing phosphonoacetate, fragmented DNA was detected from cells infected with d120 mutant but not with the wild-type. Fragmented DNA was visible in extracts of mock-infected cells incubated at 39.5°C, but not in cells infected with HSV-1tsHA1 and incubated at the same temperature.

From this series of studies, it is concluded that (i) HSV-1 is capable of inducing the morphologic and biochemical changes characteristic of apoptosis and these changes are prominent in cells infected with a mutant lacking ICP4; (ii) wild-type virus does not induce apoptosis indicating that ICP4 or a protein expressed subsequently is able to protect cells from apoptosis; (iii) the protective, anti-apoptotic effect is a viral function which does not depend on

the onset of viral DNA synthesis; (iv) DNA degradation typical of apoptosis was observed upon incubation at 39.5°C in mock-infected but not HSV-1tsHA1 infected cells, which suggests that prolonged incubation at the elevated temperature can induce apoptosis that is blocked by a viral function expressed early.

EXAMPLE 3

ICP4 expresses an anti-apoptotic function.

In a fourth series of studies, Vero or E5 cells were mock infected or infected with 10 PFU per cell with either HSV-1(F) or d120. The cells were incubated at 39.5°C for 30 hrs. The rationale of these studies was as follows. As noted in Example 1, HSV-1(F) carries a ts lesion in the α4 gene and at the nonpermissive temperature (39.5°C) expresses only α genes. The α4 gene resident in the E5 cell line and the d120 mutant virus lacking the α4 gene were derived from the HSV-1(KOS) strain which does not exhibit the *ts* phenotype. In addition, the α4 gene resident in the E5 cell line is induced after infection and is not expressed in uninfected cells. In the first series of studies, the cells were harvested, lysed, centrifuged to sediment chromosomal DNA and the supernatant fluids were processed as described in Example 1 and subjected to electrophoresis in agarose gels.

The results were as follows. Fragmented DNA was present in lanes containing electrophoretically separated extracts of mock-infected Vero cells, Vero cells infected with d120 mutant, and the mock infected E5 cells. Fragmented DNA was not detected in Vero cells infected with wild-type virus, or in E5 cells infected with either d120 mutant virus or HSV-1(F) virus.

In the second series of studies Vero cells were mock infected, or infected with either d120 or with wild-type virus. After 30 hrs of incubation at 39.5°C, the cells were fixed and labeled with biotinylated dUTP by terminal transferase, and reacted with fluorescent avidin. Fluorescence was detected in mock-infected or infected with d120 mutant, but not in cells infected with wild-type virus.

These studies allowed permit the conclusion conclude that HSV-1(F) α4 gene encodes a function which blocks apoptosis reflected in the degradation of DNA, and that this function is

separable from the repressor and transactivator functions of ICP4 which are affected by the temperature sensitive lesion of the α4 gene of HSV-1(F).

A summary of the results is provided in Table 4. Induction of (+), or protection from (-) apoptosis is indicated upon conditions (infecting virus and incubation temperature) which induce apoptosis in Vero and E5 cell lines. "nt" indicates not tested.

TABLE 4

	VERO (37°C)	VERO (39°C)	E5 (37°C)	E5 (39.5°C)
MOCK	-	+	-	+
HSV-1(F)	-	-	-	-
HSV-1 d120	+	+	-	-
HSV-1 tsHA1	-	-	nt	-

EXAMPLE 4

HSV-1(KOS)d120 carries an additional mutation.

The results described in this section emerged from studies designed to repair the deletion in both copies of the α4 gene of HSV-1(KOS)d120. The inventors isolated recombinant in which this gene was repaired by two different procedures. In the first, the inventors cloned an HSV-1(F) DNA fragment that contains an α4 sequence plus enough flanking sequence to allow homologous recombination. In this series of studies Vero cells were transfected with plasmid DNA and infected with 0.1 to 1 PFU of HSV-1(KOS)d120 per cell. Under these conditions, plaques formed only in cultures of cells transfected with the plasmid DNA. Recombinant virus was recovered from individual plaques, and designated 120FR [for HSV-1(F) repair].

The second procedure was based on the observation that a small amount of virus recombines with the resident α4 gene in the E5 cell line to yield rescued virus capable of replicating efficiently in the absence of exogenous source of ICP4. The observed recombination frequency is 10^{-6} to 10^{-7} and therefore such rescued virus would be expected to be present in HSV-1(KOS)d120 stock. To isolate these recombinants, Vero cells were infected

at a high multiplicity with HSV-1 d120. At 24 hr after infection cells were harvested, frozen-thawed and serial dilutions were used to infect Vero cells. The recombinant virus obtained in this fashion was designated 120KR [for HSV-1(KOS) repair].

As shown in FIG. 3, analyses of DNA extracted from replicate Vero cell cultures infected with HSV-1(KOS)d120, 120FR or 120KR showed ladders typical of apopotic cells. These ladders were absent in extracts of mock-infected cells or cells infected with HSV-1(F). The inventors conclude from these studies that HSV-1(KOS)d120 genome contain an additional mutation other than in the α4 gene.

EXAMPLE 5

The U_S3 protein kinase is required to block apoptosis induced by HSV-1 infection.

In the following series of studies, the inventors defined the region that contains the additional mutation in HSV-1(KOS)d120 by rescue of the HSV-1(KOS)d120 with cosmids containing large HSV-1 DNA fragments. The three cosmids used in these studies were cosmid pBC8008, which contains all of the HSV-1 terminal repeat sequence, almost all of the U_S sequence, and part of the U_L sequence (FIG. 1) and cosmids pBC8004 and pBC8009 which contain fragments spanning over the entire repeat sequence but differ in the extent of coverage of the U_S region. Individual plaques purified from each transfection were tested for their ability to protect infected cells from apoptosis induced by infection. The results shown in Table 5 suggested that the second mutation in HSV-1(KOS)d120 may map in the U_S domain containing the genes U_S1 through U_S3.

TABLE 5

Plasmid/Cosmid	Recombinant	Protection From Apoptosis
pRB5166	HSV-1 120FR	0/9
none	HSV-1 120KR	0/5
pBC1004	HSV-1 120AR	1/4
pBC1009	HSV-1 120BR	0/9
pBC1008	HSV-1 120CR	8/9

To map the function required to block apoptosis more precisely, the inventors took advantage of the availability in this laboratory of several deletion mutants which span the sequence thought to encode the gene required to block apoptosis in infected cells. Recombinant virus R325 lacks the carboxyl terminal half of the α22 gene, and the 3′ domain of the U_S2 gene. Recombinant virus R7041, lacks most of the U_S3 gene whereas in recombinant R7306 the U_S3 gene had been repaired. The results, shown in FIG. 3 indicate that apoptosis was induced in cells infected with R7041 (ΔU_S3) but not in cells infected with the other mutants.

Two series of studies verified the conclusion that a functional U_S3 gene is required for prevention of apoptosis and that the second mutation in the HSV-1(KOS)d120 resides in the U_S3 gene. In the first series, the inventors carried out simple complementation tests to determine whether the second mutation in HSV-1(KOS)d120 was the same as that in the rescued virus or in R7041 (ΔU_S3) recombinant. Specifically Vero cells were infected with artificial mixtures of 120FR and HSV-1(KOS)d120, 120FR and R7041, or 120FR and R7306 (U_S3 repaired). The results, shown in FIG. 4 indicate the following. As could be expected, 120FR did not complement HSV-1(KOS)d120, the parent virus from which it was derived. The second, mixture of 120FR and R7041 also did not complement each other suggesting that 120FR and its parent virus, HSV-1(KOS)d120 contained a nonfunctional U_S3. Verification of this hypothesis emerged from the observation that R7306 containing a repaired U_S3 gene complemented 120FR and blocked apoptosis in cells infected with these two viruses.

The second series of studies served to verify the HSV-1(KOS)d120 and its derivatives lacked a functional U_S3 gene. As described in this introduction, the production of U_L34 gene

has been shown in earlier studies from this laboratory to be a substrate for the U_S3 protein kinase. In wild type infected cells, U_L34 has an apparent M_r of 30,000 whereas in cells infected with R7041 (ΔU_S3), U_L34 has an apparent migrates of M_r 33,000. Inasmuch as the original studies were done in BHK cells, in this series of studies replicate cultures of BHK cells were infected 10 PFU of HSV-1(F), 120FR, R7041, and R7306 per cell. The results, shown in FIG. 5 indicate that the fully processed U_L34 protein was present in cells infected with HSV-1(F) but not cells infected with 120FR or R7041. As could be expected, the cells infected with HSV-1(KOS)d120 did not make detectable quantities of U_L34 protein inasmuch as they lack the $\alpha 4$ gene. In the absense of $\alpha 4$ gene expression there is no β or γ gene expression and therefore, no U_S3 gene expression. Taken together, the inventors' results indicate the U_S3 kinase is functionally absent in HSV-1(KOS)d120 and 120FR. The inventors conclude that U_S3 is required for protection from apoptosis induced by HSV-1.

XI. References

The following references, to the extent that they provide exemplary procedural details or other information supplementary to that set forth herein, are incorporated by reference:

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Zhu *et al.*, *J. Virol.*, 69:7960-7970, 1995.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:
(A) NAME: Arch Development Corporation
(B) STREET: 1101 East 58th Street
(C) CITY: Chicago
(D) STATE: IL
(E) COUNTRY: USA
(F) POSTAL CODE (ZIP): 60637
(G) TELEPHONE: 512-418-3000
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(ii) TITLE OF INVENTION: HERPES SIMPLEX VIRUS U_S3 AND ICP4 AS INHIBITORS OF APOPTOSIS

(iii) NUMBER OF SEQUENCES: 6:

(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)

(v) CURRENT APPLICATION DATA:
APPLICATION NUMBER: US 08/843,659

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 4257 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

GCTGCTCCTC CTTCCCGCCG GCCCCTGGGA CTATATGAGC CCGAGGACGC CCCGATCGTC 60

CACACGGAGC GCGGCTGCCG ACACGGATCC ACGACCCGAC GCGGGACCGC CAGAGACAGA 120

CCGTCAGACG CTCGCCGCGC CGGGACGCCG ATACGCGGAC GAAGCGCGGG AGGGGGATCG 180

GCCGTCCCTG TCCTTTTTCC CACCCAAGCA TCGACCGGTC CGCGCTAGTT CCGCGTCGAC 240

GGCGGGGGTC GTCGGGGTCC GTGGGTCTCG CCCCCTCCCC CCATCGAGAG TCCGTAGGTG 300

ACCTACCGTG CTACGTCCGC CGTCGCAGCC GTATCCCCGG AGGATCGCCC CGCATCGGCG 360

ATGGCGTCGG AGAACAAGCA GCGCCCCGGC TCCCCGGGCC CCACCGACGG GCCGCCGCCC 420

ACCCCGAGCC CAGACCGCGA CGAGCGGGGG GCCCTCGGGT GGGGCGCGGA GACGGAGGAG 480

GGCGGGGACG ACCCCGACCA CGACCCCGAC CACCCCCACG ACCTCGACGA CGCCCGGCGG 540

GACGGGAGGG CCCCCGCGGC GGGCACCGAC GCCGGCGAGG ACGCCGGGGA CGCCGTCTCG 600

CCGCGACAGC TGGCTCTGCT GGCCTCCATG GTAGAGGAGG CCGTCCGGAC GATCCCGACG 660

CCCGACCCCG CGGCCTCGCC GCCCCGGACC CCCGCCTTTC GAGCCGACGA CGATGACGGG 720

GACGAGTACG ACGACGCAGC CGACGCCGCC GGCGACCGGG CCCCGGCCCG GGGCCGCGAA 780

CGGGAGGCCC CGCTACGCGG CGCGTATCCG GACCCCACGG ACCGCCTGTC GCCGCGCCCG 840

CCGGCCCAGC CGCCGCGGAG ACGTCGTCAC GGCCGGTGGC GGCCATCGGC GTCATCGACC 900

TCGTCGGACT CCGGGTCCTC GTCCTCGTCG TCCGCATCCT CTTCGTCCTC GTCGTCCGAC 960

GAGGACGAGG ACGACGACGG CAACGACGCG GCCGACCACG CACGCGAGGC GCGGGCCGTC 1020

GGGCGGGGTC CGTCGAGCGC GGCGCCGGCA GCCCCCGGGC GGACGCCGCC CCCGCCCGGG 1080

CCACCCCCCC TCTCCGAGGC CGCGCCCAAG CCCCGGGCGG CGGCGAGGAC CCCCGCGGCC 1140

TCCGCGGGCC GCATCGAGCG CCGCCGGGCC CGCGCGGCGG TGGCCGGCCG CGACGCCACG 1200

GGCCGCTTCA CGGCCGGGCA GCCCCGGCGG GTCGAGCTGG ACGCCGACGC GACCTCCGGC 1260

GCCTTCTACG CGCGCTATCG CGACGGGTAC GTCAGCGGGG AGCCGTGGCC CGGCGCCGGG 1320

CCCCCGCCCC CGGGGCGGGT GCTGTACGGC GGCCTGGGCG ACAGCCGCCC GGGCCTCTGG 1380

GGGGCGCCCG AGGCGGAGGA GGCGCGACGC CGGTTCGAGG CCTCGGGCGC CCCGGCGGCC 1440

GTGTGGGCGC CCGAGCTGGG CGACGCCGCG CAGCAGTACG CCCTGATCAC GCGGCTGCTG 1500

TACACCCCGG ACGCGGAGGC CATGGGGTGG CTCCAGAACC CGCGCGTGGT CCCCGGGGAC 1560

GTGGCGCTGG ACCAGGCCTG CTTCCGGATC TCGGGCGCCG CGCGCAACAG CAGCTCCTTC 1620

ATCACCGGCA GCGTGGCGCG GGCCGTGCCC CACCTGGGCT ACGCCATGGC GGCCGGCCGC 1680

TTCGGCTGGG GCCTGGCGCA CGCGGCGGCC GCCGTGGCCA TGAGCCGCCG ATACGACCGC 1740

GCGCAGAAGG GCTTCCTGCT GACCAGCCTG CGCCGCGCCT ACGCGCCCCT GTTGGCGCGC 1800

GAGAACGCGG CGCTGACGGG GGCCGCGGGG AGCCCCGGCG CCGGCGCAGA TGACGAGGGG 1860

GTCGCCGCCG TCGCCGCCGC CGCACCGGGC GAGCGCGCGG TGCCCGCCGG GTACGGCGCC 1920

GCGGGGATCC TCGCCGCCCT GGGGCGGCTG TCCGCCGCGC CCGCCTCCCC CGCGGGGGGC 1980

GACGACCCCG ACGCCGCCCG CCACGCCGAC GCCGACGACG ACGCCGGGCG CCGCGCCCAG 2040

GCCGGCCGCG TGGCCGTCGA GTGCCTGGCC GCCTGCCGCG GGATCCTGGA GGCGCTGGCC 2100

GAGGGCTTCG ACGGCGACCT GGCGGCCGTC CCGGGGCTGG CCGGGGCCCG GCCCGCCAGC 2160

CCCCCGCGGC CGGAGGGACC CGCGGGCCCC GCTTCCCCGC CGCCGCCGCA CGCCGACGCG 2220

CCCCGCCTGC GCGCGTGGCT GCGCGAGCTG CGGTTCGTGC GCGACGCGCT GGTGCTCATG 2280

CGCCTGCGCG GGGACCTGCG CGTGGCCGGC GGCAGCGAGG CCGCCGTGGC CGCCGTGCGC 2340

GCCGTGAGCC TGGTCGCCGG GGCCCTGGGC CCCGCGCTGC CGCGGGACCC GCGCCTGCCG 2400

AGCTCCGCGG CCGCCGCCGC CGCGGACCTG CTGTTTGACA ACCAGAGCCT GCGCCCCCTG 2460

CTGGCGGCGG CGGCCAGCGC ACCGGACGCC GCCGACGCGC TGGCGGCCGC CGCCGCCTCC 2520

GCCGCGCCGC GGGAGGGGCG CAAGCGCAAG AGTCCCGGCC CGGCCCGGCC GCCCGGAGGC 2580

GGCGGCCCGC GACCCCCGAA GACGAAGAAG AGCGGCGCGG ACGCCCCCGG CTCGGACGCC 2640

CGCGCCCCCC TCCCCGCGCC CGCGCCCCCC TCCACGCCCC CGGGGCCCGA GCCCGCCCCC 2700

GCCCAGCCCG CGGCGCCCCG GGCCGCCGCG GCGCAGGCCC GCCCGCGCCC CGTGGCCGTG 2760

TCGCGCCGGC CCGCCGAGGG CCCCGACCCC CTGGGCGGCT GGCGGCGGCA GCCCCCGGGG 2820

CCCAGCCACA CGGCGGCGCC CGCGGCCGCC GCCCTGGAGG CCTACTGCTC CCCGCGCGCC 2880

GTGGCCGAGC TCACGGACCA CCCGCTGTTC CCCGTCCCCT GGCGACCGGC CCTCATGTTT 2940

GACCCGCGGG CCCTGGCCTC GATCGCCGCG CGGTGCGCCG GGCCCGCCCC CGCCGCCCAG 3000

GCCGCGTGCG GCGGCGGCGA CGACGACGAT AACCCCCACC CCCACGGGGC CGCCGGGGGC 3060

CGCCTCTTTG GCCCCCTGCG CGCCTCGGGC CCGCTGCGCC GCATGGCGGC CTGGATGCGC 3120

CAGATCCCCG ACCCCGAGGA CGTGCGCGTG GTGGTGCTGT ACTCGCCGCT GCCGGGCGAG 3180

GACCTGGCCG GCGGCGGGGC CTCGGGGGGG CCGCCGGAGT GGTCCGCCGA GCGCGGCGGG 3240

CTGTCCTGCC TGCTGGCGGC CCTGGCCAAC CGGCTGTGCG GGCCGGACAC GGCCGCCTGG 3300

GCGGGCAATT GGACCGGCGC CCCCGACGTG TCGGCGCTGG GCGCACAGGG CGTGCTGCTG 3360

CTGTCCACGC GGGACCTGGC CTTCGCCGGG GCCGTGGAGT TTCTGGGGCT GCTCGCCAGC 3420

GCCGGCGACC GGCGGCTCAT CGTGGTCAAC ACCGTGCGCG CCTGCGACTG GCCCGCCGAC 3480

GGGCCCGCGG TGTCGCGGCA GCACGCCTAC CTGGCGTGCG AGCTGCTGCC CGCCGTGCAG 3540

TGCGCCGTGC GCTGGCCGGC GGCGCGGGAC CTGCGCCGCA CGGTGCTGGC CTCGGGCCGC 3600

GTGTTCGGCC CGGGGGTCTT CGCGCGCGTG GAGGCCGCGC ACGCGCGCCT GTACCCCGAC 3660

GCGCCGCCGC TGCGCCTGTG CCGCGGCGGC AACGTGCGCT ACCGCGTGCG CACGCGCTTC 3720

GGCCCGGACA CGCCGGTGCC CATGTCCCCG CGCGAGTACC GCCGGGCCGT GCTGCCGGCG 3780

CTGGACGGCC GGGCGGCGGC CTCGGGGACC ACCGACGCCA TGGCGCCCGG CGCGCCGGAC 3840

TTCTGCGAGG AGGAGGCCCA CTCGCACGCC GCCTGCGCGC GCTGGGGCCT GGGCGCGCCG 3900

CTGCGGCCCG TGTACGTGGC GCTGGGGCGC GAGGCGGTGC GCGCCGGCCC GGCCCGGTGG 3960

CGCGGGCCGC GGAGGGACTT TTGCGCCCGC GCCCTGCTGG AGCCCGACGA CGACGCCCCC 4020

CCGCTGGTGC TGCGCGGCGA CGACGACGGC CCGGGGGCCC TGCCGCCGGC GCCGCCCGGG 4080

ATTCGCTGGG CCTCGGCCAC GGGCCGCAGC GGCACCGTGC TGGCGGCGGC GGGGGCCGTG 4140

GAGGTGCTGG GGGCGGAGGC GGGCTTGGCC ACGCCCCCGC GGCGGGAAGT TGTGGACTGG 4200

GAAGGCGCCT GGGACGAAGA CGACGGCGGC GCGTTCGAGG GGGACGGGGT GCTGTAA 4257

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1298 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

Met Ala Ser Glu Asn Lys Gln Arg Pro Gly Ser Pro Gly Pro Thr Asp
1 5 10 15

Gly Pro Pro Pro Thr Pro Ser Pro Asp Arg Asp Glu Arg Gly Ala Leu
20 25 30

Gly Trp Gly Ala Glu Thr Glu Glu Gly Gly Asp Asp Pro Asp His Asp
35 40 45

Pro Asp His Pro His Asp Leu Asp Asp Ala Arg Arg Asp Gly Arg Ala
50 55 60

Pro Ala Ala Gly Thr Asp Ala Gly Glu Asp Ala Gly Asp Ala Val Ser
65 70 75 80

Pro Arg Gln Leu Ala Leu Leu Ala Ser Met Val Glu Glu Ala Val Arg
85 90 95

Thr Ile Pro Thr Pro Asp Pro Ala Ala Ser Pro Pro Arg Thr Pro Ala
100 105 110

Phe Arg Ala Asp Asp Asp Asp Gly Asp Glu Tyr Asp Asp Ala Ala Asp
115 120 125

Ala Ala Gly Asp Arg Ala Pro Ala Arg Gly Arg Glu Arg Glu Ala Pro
130 135 140

Leu Arg Gly Ala Tyr Pro Asp Pro Thr Asp Arg Leu Ser Pro Arg Pro
145 150 155 160

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Pro Ala Gln Pro Pro Arg Arg Arg Arg His Gly Arg Trp Arg Pro Ser
                165                 170                 175

Ala Ser Ser Thr Ser Ser Asp Ser Gly Ser Ser Ser Ser Ser Ser Ala
            180                 185                 190

Ser Ser Ser Ser Ser Ser Ser Asp Glu Asp Glu Asp Asp Asp Gly Asn
        195                 200                 205

Asp Ala Ala Asp His Ala Arg Glu Ala Arg Ala Val Gly Arg Gly Pro
    210                 215                 220

Ser Ser Ala Ala Pro Ala Ala Pro Gly Arg Thr Pro Pro Pro Pro Gly
225                 230                 235                 240

Pro Pro Pro Leu Ser Glu Ala Ala Pro Lys Pro Arg Ala Ala Ala Arg
                245                 250                 255

Thr Pro Ala Ala Ser Ala Gly Arg Ile Glu Arg Arg Arg Ala Arg Ala
            260                 265                 270

Ala Val Ala Gly Arg Asp Ala Thr Gly Arg Phe Thr Ala Gly Gln Pro
        275                 280                 285

Arg Arg Val Glu Leu Asp Ala Asp Ala Thr Ser Gly Ala Phe Tyr Ala
    290                 295                 300

Arg Tyr Arg Asp Gly Tyr Val Ser Gly Glu Pro Trp Pro Gly Ala Gly
305                 310                 315                 320

Pro Pro Pro Pro Gly Arg Val Leu Tyr Gly Gly Leu Gly Asp Ser Arg
                325                 330                 335

Pro Gly Leu Trp Gly Ala Pro Glu Ala Glu Glu Ala Arg Arg Arg Phe
            340                 345                 350

Glu Ala Ser Gly Ala Pro Ala Ala Val Trp Ala Pro Glu Leu Gly Asp
        355                 360                 365

Ala Ala Gln Gln Tyr Ala Leu Ile Thr Arg Leu Leu Tyr Thr Pro Asp
    370                 375                 380

Ala Glu Ala Met Gly Trp Leu Gln Asn Pro Arg Val Val Pro Gly Asp
385                 390                 395                 400

Val Ala Leu Asp Gln Ala Cys Phe Arg Ile Ser Gly Ala Ala Arg Asn
                405                 410                 415

Ser Ser Ser Phe Ile Thr Gly Ser Val Ala Arg Ala Val Pro His Leu
            420                 425                 430

Gly Tyr Ala Met Ala Ala Gly Arg Phe Gly Trp Gly Leu Ala His Ala
        435                 440                 445

Ala Ala Ala Val Ala Met Ser Arg Arg Tyr Asp Arg Ala Gln Lys Gly
    450                 455                 460
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Phe Leu Leu Thr Ser Leu Arg Arg Ala Tyr Ala Pro Leu Leu Ala Arg
465                 470                 475                 480

Glu Asn Ala Ala Leu Thr Gly Ala Ala Gly Ser Pro Gly Ala Gly Ala
                485                 490                 495

Asp Asp Glu Gly Val Ala Ala Val Ala Ala Ala Ala Pro Gly Glu Arg
            500                 505                 510

Ala Val Pro Ala Gly Tyr Gly Ala Ala Gly Ile Leu Ala Ala Leu Gly
        515                 520                 525

Arg Leu Ser Ala Ala Pro Ala Ser Pro Ala Gly Gly Asp Asp Pro Asp
    530                 535                 540

Ala Ala Arg His Ala Asp Ala Asp Asp Asp Ala Gly Arg Arg Ala Gln
545                 550                 555                 560

Ala Gly Arg Val Ala Val Glu Cys Leu Ala Ala Cys Arg Gly Ile Leu
                565                 570                 575

Glu Ala Leu Ala Glu Gly Phe Asp Gly Asp Leu Ala Ala Val Pro Gly
            580                 585                 590

Leu Ala Gly Ala Arg Pro Ala Ser Pro Pro Arg Pro Glu Gly Pro Ala
        595                 600                 605

Gly Pro Ala Ser Pro Pro Pro Pro His Ala Asp Ala Pro Arg Leu Arg
    610                 615                 620

Ala Trp Leu Arg Glu Leu Arg Phe Val Arg Asp Ala Leu Val Leu Met
625                 630                 635                 640

Arg Leu Arg Gly Asp Leu Arg Val Ala Gly Gly Ser Glu Ala Ala Val
                645                 650                 655

Ala Ala Val Arg Ala Val Ser Leu Val Ala Gly Ala Leu Gly Pro Ala
            660                 665                 670

Leu Pro Arg Asp Pro Arg Leu Pro Ser Ser Ala Ala Ala Ala Ala Ala
        675                 680                 685

Asp Leu Leu Phe Asp Asn Gln Ser Leu Arg Pro Leu Leu Ala Ala Ala
    690                 695                 700

Ala Ser Ala Pro Asp Ala Ala Asp Ala Leu Ala Ala Ala Ala Ala Ser
705                 710                 715                 720

Ala Ala Pro Arg Glu Gly Arg Lys Arg Lys Ser Pro Gly Pro Ala Arg
                725                 730                 735

Pro Pro Gly Gly Gly Gly Pro Arg Pro Pro Lys Thr Lys Lys Ser Gly
            740                 745                 750
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Ala Asp Ala Pro Gly Ser Asp Ala Arg Ala Pro Leu Pro Ala Pro Ala
        755                 760                 765

Pro Pro Ser Thr Pro Pro Gly Pro Glu Pro Ala Pro Ala Gln Pro Ala
    770                 775                 780

Ala Pro Arg Ala Ala Ala Ala Gln Ala Arg Pro Arg Pro Val Ala Val
785                 790                 795                 800

Ser Arg Arg Pro Ala Glu Gly Pro Asp Pro Leu Gly Gly Trp Arg Arg
                805                 810                 815

Gln Pro Pro Gly Pro Ser His Thr Ala Ala Pro Ala Ala Ala Ala Leu
            820                 825                 830

Glu Ala Tyr Cys Ser Pro Arg Ala Val Ala Glu Leu Thr Asp His Pro
        835                 840                 845

Leu Phe Pro Val Pro Trp Arg Pro Ala Leu Met Phe Asp Pro Arg Ala
    850                 855                 860

Leu Ala Ser Ile Ala Ala Arg Cys Ala Gly Pro Ala Pro Ala Ala Gln
865                 870                 875                 880

Ala Ala Cys Gly Gly Gly Asp Asp Asp Asp Asn Pro His Pro His Gly
                885                 890                 895

Ala Ala Gly Gly Arg Leu Phe Gly Pro Leu Arg Ala Ser Gly Pro Leu
            900                 905                 910

Arg Arg Met Ala Ala Trp Met Arg Gln Ile Pro Asp Pro Glu Asp Val
        915                 920                 925

Arg Val Val Val Leu Tyr Ser Pro Leu Pro Gly Glu Asp Leu Ala Gly
    930                 935                 940

Gly Gly Ala Ser Gly Gly Pro Pro Glu Trp Ser Ala Glu Arg Gly Gly
945                 950                 955                 960

Leu Ser Cys Leu Leu Ala Ala Leu Ala Asn Arg Leu Cys Gly Pro Asp
                965                 970                 975

Thr Ala Ala Trp Ala Gly Asn Trp Thr Gly Ala Pro Asp Val Ser Ala
            980                 985                 990

Leu Gly Ala Gln Gly Val Leu Leu Leu Ser Thr Arg Asp Leu Ala Phe
        995                 1000                1005

Ala Gly Ala Val Glu Phe Leu Gly Leu Leu Ala Ser Ala Gly Asp Arg
    1010                1015                1020

Arg Leu Ile Val Val Asn Thr Val Arg Ala Cys Asp Trp Pro Ala Asp
1025                1030                1035                1040

Gly Pro Ala Val Ser Arg Gln His Ala Tyr Leu Ala Cys Glu Leu Leu
                1045                1050                1055
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Pro Ala Val Gln Cys Ala Val Arg Trp Pro Ala Ala Arg Asp Leu Arg
1060 1065 1070

Arg Thr Val Leu Ala Ser Gly Arg Val Phe Gly Pro Gly Val Phe Ala
1075 1080 1085

Arg Val Glu Ala Ala His Ala Arg Leu Tyr Pro Asp Ala Pro Pro Leu
1090 1095 1100

Arg Leu Cys Arg Gly Gly Asn Val Arg Tyr Arg Val Arg Thr Arg Phe
1105 1110 1115 1120

Gly Pro Asp Thr Pro Val Pro Met Ser Pro Arg Glu Tyr Arg Arg Ala
1125 1130 1135

Val Leu Pro Ala Leu Asp Gly Arg Ala Ala Ala Ser Gly Thr Thr Asp
1140 1145 1150

Ala Met Ala Pro Gly Ala Pro Asp Phe Cys Glu Glu Glu Ala His Ser
1155 1160 1165

His Ala Ala Cys Ala Arg Trp Gly Leu Gly Ala Pro Leu Arg Pro Val
1170 1175 1180

Tyr Val Ala Leu Gly Arg Glu Ala Val Arg Ala Gly Pro Ala Arg Trp
1185 1190 1195 1200

Arg Gly Pro Arg Arg Asp Phe Cys Ala Arg Ala Leu Leu Glu Pro Asp
1205 1210 1215

Asp Asp Ala Pro Pro Leu Val Leu Arg Gly Asp Asp Asp Gly Pro Gly
1220 1225 1230

Ala Leu Pro Pro Ala Pro Pro Gly Ile Arg Trp Ala Ser Ala Thr Gly
1235 1240 1245

Arg Ser Gly Thr Val Leu Ala Ala Ala Gly Ala Val Glu Val Leu Gly
1250 1255 1260

Ala Glu Ala Gly Leu Ala Thr Pro Pro Arg Arg Glu Val Val Asp Trp
1265 1270 1275 1280

Glu Gly Ala Trp Asp Glu Asp Asp Gly Gly Ala Phe Glu Gly Asp Gly
1285 1290 1295

Val Leu

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1446 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

ATGGCCTGTC GTAAGTTTTG TCGCGTTTAC GGGGGACAGG GCAGGAGGAA GGAGGAGGCC 60

GTCCCGCCGG AGACAAAGCC GTCCCGGGTG TTTCCTCATG GCCCCTTTTA TACCCCAGCC 120

GAGGACGCGT GCCTGGACTC CCCGCCCCCG GAGACCCCCA AACCTTCCCA CACCACACCA 180

CCCAGCGAGG CCGAGCGCCT GTGTCATCTG CAGGAGATCC TTGCCCAGAT GTACGGAAAC 240

CAGGACTACC CCATAGAGGA CGACCCCAGC GCGGATGCCG CGGACGATGT CGACGAGGAC 300

GCCCCGGACG ACGTGGCCTA TCCGGAGGAA TACGCAGAGG AGCTTTTTCT GCCCGGGGAC 360

GCGACCGGTC CCCTTATCGG GGCCAACGAC CACATCCCTC CCCCGTGTGG CGCATCTCCC 420

CCCGGTATAC GACGACGCAG CCGGGATGAG ATTGGGGCCA CGGGATTTAC CGCGGAAGAG 480

CTGGACGCCA TGGACAGGGA GGCGGCTCGA GCCATCAGCC GCGGCGGCAA GCCCCCCTCG 540

ACCATGGCCA AGCTGGTGAC TGGCATGGGC TTTACGATCC ACGGAGCGCT CACCCCAGGA 600

TCGGAGGGGT GTGTCTTTGA CAGCAGCCAT CCAGATTACC CCCAACGGGT AATCGTGAAG 660

GCGGGGTGGT ACACGAGCAC GAGCCACGAG GCGCGACTGC TGAGGCGACT GGACCACCCG 720

GCGATCCTGC CCCTCCTGGA CCTGCATGTC GTCTCCGGGG TCACGTGTCT GGTCCTCCCC 780

AAGTACCAGG CCGACCTGTA TACCTATCTG AGTAGGCGCC TGAACCCACT GGGACGCCCG 840

CAGATCGCAG CGGTCTCCCG GCAGCTCCTA AGCGCCGTTG ACTACATTCA CCGCCAGGGC 900

ATTATCCACC GCGACATTAA GACCGAAAAT ATTTTTATTA ACACCCCCGA GGACATTTGC 960

CTGGGGGACT TTGGCGCCGC GTGCTTCGTG CAGGGTTCCC GATCAAGCCC CTTCCCCTAC 1020

GGAATCGCCG GAACCATCGA CACCAACGCC CCCGAGGTCC TGGCCGGGGA TCCGTATACC 1080

ACGACCGTCG ACATTTGGAG CGCCGGTCTG GTGATCTTCG AGACTGCCGT CCACAACGCG 1140

TCCTTGTTCT CGGCCCCCCG CGGCCCCAAA AGGGGCCCGT GCGACAGTCA GATCACCCGC 1200

ATCATCCGAC AGGCCCAGGT CCACGTTGAC GAGTTTTCCC CGCATCCAGA ATCGCGCCTC 1260

ACCTCGCGCT ACCGCTCCCG CGCGGCCGGG AACAATCGCC CGCCGTACAC CCGACCGGCC 1320

TGGACCCGCT ACTACAAGAT GGACATAGAC GTCGAATATC TGGTTTGCAA AGCCCTCACC 1380

TTCGACGGCG CGCTTCGCCC CAGCGCCGCA GAGCTGCTTT GTTTGCCGCT GTTTCAACAG 1440

AAATGA 1446

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 481 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

Met Ala Cys Arg Lys Phe Cys Arg Val Tyr Gly Gly Gln Gly Arg Arg
1 5 10 15

Lys Glu Glu Ala Val Pro Pro Glu Thr Lys Pro Ser Arg Val Phe Pro
20 25 30

His Gly Pro Phe Tyr Thr Pro Ala Glu Asp Ala Cys Leu Asp Ser Pro
35 40 45

Pro Pro Glu Thr Pro Lys Pro Ser His Thr Thr Pro Pro Ser Glu Ala
50 55 60

Glu Arg Leu Cys His Leu Gln Glu Ile Leu Ala Gln Met Tyr Gly Asn
65 70 75 80

Gln Asp Tyr Pro Ile Glu Asp Asp Pro Ser Ala Asp Ala Ala Asp Asp
85 90 95

Val Asp Glu Asp Ala Pro Asp Asp Val Ala Tyr Pro Glu Glu Tyr Ala
100 105 110

Glu Glu Leu Phe Leu Pro Gly Asp Ala Thr Gly Pro Leu Ile Gly Ala
115 120 125

Asn Asp His Ile Pro Pro Pro Cys Gly Ala Ser Pro Pro Gly Ile Arg
130 135 140

Arg Arg Ser Arg Asp Glu Ile Gly Ala Thr Gly Phe Thr Ala Glu Glu
145 150 155 160

Leu Asp Ala Met Asp Arg Glu Ala Ala Arg Ala Ile Ser Arg Gly Gly
165 170 175

Lys Pro Pro Ser Thr Met Ala Lys Leu Val Thr Gly Met Gly Phe Thr
180 185 190

Ile His Gly Ala Leu Thr Pro Gly Ser Glu Gly Cys Val Phe Asp Ser
195 200 205

Ser His Pro Asp Tyr Pro Gln Arg Val Ile Val Lys Ala Gly Trp Tyr
210 215 220

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Thr Ser Thr Ser His Glu Ala Arg Leu Leu Arg Arg Leu Asp His Pro
225                 230                 235                 240

Ala Ile Leu Pro Leu Leu Asp Leu His Val Val Ser Gly Val Thr Cys
                245                 250                 255

Leu Val Leu Pro Lys Tyr Gln Ala Asp Leu Tyr Thr Tyr Leu Ser Arg
            260                 265                 270

Arg Leu Asn Pro Leu Gly Arg Pro Gln Ile Ala Ala Val Ser Arg Gln
        275                 280                 285

Leu Leu Ser Ala Val Asp Tyr Ile His Arg Gln Gly Ile Ile His Arg
    290                 295                 300

Asp Ile Lys Thr Glu Asn Ile Phe Ile Asn Thr Pro Glu Asp Ile Cys
305                 310                 315                 320

Leu Gly Asp Phe Gly Ala Ala Cys Phe Val Gln Gly Ser Arg Ser Ser
                325                 330                 335

Pro Phe Pro Tyr Gly Ile Ala Gly Thr Ile Asp Thr Asn Ala Pro Glu
            340                 345                 350

Val Leu Ala Gly Asp Pro Tyr Thr Thr Thr Val Asp Ile Trp Ser Ala
        355                 360                 365

Gly Leu Val Ile Phe Glu Thr Ala Val His Asn Ala Ser Leu Phe Ser
    370                 375                 380

Ala Pro Arg Gly Pro Lys Arg Gly Pro Cys Asp Ser Gln Ile Thr Arg
385                 390                 395                 400

Ile Ile Arg Gln Ala Gln Val His Val Asp Glu Phe Ser Pro His Pro
                405                 410                 415

Glu Ser Arg Leu Thr Ser Arg Tyr Arg Ser Arg Ala Ala Gly Asn Asn
            420                 425                 430

Arg Pro Pro Tyr Thr Arg Pro Ala Trp Thr Arg Tyr Tyr Lys Met Asp
        435                 440                 445

Ile Asp Val Glu Tyr Leu Val Cys Lys Ala Leu Thr Phe Asp Gly Ala
    450                 455                 460

Leu Arg Pro Ser Ala Ala Glu Leu Leu Cys Leu Pro Leu Phe Gln Gln
465                 470                 475                 480

Lys
```

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

Arg Arg Arg Arg Thr Arg Arg Ser Arg Glu
1 5 10

(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 6 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION:4
(D) OTHER INFORMATION:/note= "Residue may be arginine or any amino acid residue."

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION:5
(D) OTHER INFORMATION:/note= "Residue may be either serine or threonine."

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION:6
(D) OTHER INFORMATION:/note= "Residue may be either arginine or tyrosine."

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

Arg Arg Arg Arg Ser Arg
1 5

CLAIMS

1. A method for blocking apoptosis of a cell comprising the step of providing to said cell an HSV U_S3 polypeptide.

2. The method of claim 1, wherein said providing comprises contacting said cell with said HSV U_S3 polypeptide.

3. The method of claim 1, wherein said providing comprises contacting said cell with an HSV U_S3 gene.

4. The method of claim 3, wherein said gene is contained in an expression vector.

5. The method of claim 4, wherein said gene is under the transcriptional control of a promoter active in eukaryotic cells.

6. The method of claim 5, wherein said promoter is a tetracycline controlled promoter.

7. The method of claim 6, wherein said expression vector further comprises a selectable marker.

8. The method of claim 7, wherein said expression vector further comprises a gene encoding a second polypeptide under the transcriptional control of a promoter active in eukaryotic cells.

9. The method of claim 8, wherein said second gene encodes an HSV ICP4 polypeptide.

10. A method for inducing apoptosis in a cell infected with HSV comprising the step of administering to said cell an agent that inhibits HSV U_S3 function in said cell.

11. The method of claim 10, wherein said agent inhibits transcription of an HSV U_S3 gene.

12. The method of claim 10, wherein said agent inhibits translation of an HSV U_S3 gene transcript.

13. The method of claim 10, wherein said agent binds to an HSV U_S3 polypeptide.

14. The method of claim 11, wherein said reagent is an antisense HSV U_S3 construct.

15. The method of claim 12, wherein said reagent is an antisense HSV U_S3 construct.

16. The method of claim 13, wherein said reagent is an antibody that binds immunologically to an HSV U_S3 polypeptide.

17. The method of claim 14, wherein said construct comprises an oligonucleotide that hybridizes to a 5'-untranslated region for an HSV U_S3 gene.

18. The method of claim 15, wherein said construct comprises an oligonucleotide that hybridizes to a translational start site for an HSV U_S3 transcript.

19. The method of claim 16, wherein said antibody is a monoclonal antibody.

20. The method of claim 9, further comprising the step of administering to said cell an agent that inhibits HSV ICP4 function in said cell.

21. The method of claim 20, wherein said agent inhibits translation of an HSV ICP4 gene transcript.

22. The method of claim 20, wherein said agent binds to an HSV ICP4 polypeptide.

23. The method of claim 20, wherein said agent inhibits transcription of an HSV $\alpha 4$ gene.

24. The method of claim 21, wherein said reagent is an antisense HSV ICP4 construct.

25. The method of claim 23, wherein said reagent is an antisense HSV ICP4 construct.

26. The method of claim 22, wherein said reagent is an antibody that binds immunologically to an HSV ICP4 polypeptide.

27. The method of claim 24, wherein said construct comprises an oligonucleotide that hybridizes to a 5'-untranslated region for an HSV α4 gene.

28. The method of claim 23, wherein said construct comprises an oligonucleotide that hybridizes a translational start site for an HSV ICP4 transcript.

29. The method of claim 26, wherein said antibody is a monoclonal antibody.

30. A method for treating a subject with an HSV infection comprising the step of inhibiting HSV U_S3 function.

31. The method of claim 30, wherein the inhibition comprises providing to said subject a first pharmaceutical composition comprising an HSV U_S3 antisense construct.

32. The method of claim 30, wherein the inhibition comprises providing to said subject a first pharmaceutical composition comprising a monoclonal antibody that binds immunologically to an HSV U_S3 polypeptide.

33. The method of claim 31, wherein said first pharmaceutical composition is applied topically to HSV infected cells in said patient..

34. The method of claim 32, wherein said first pharmaceutical composition is applied topically to HSV infected cells in said patient.

35. The method of claim 30, further comprising the step of inhibiting HSV ICP4 function.

36. The method of claim 30, further comprising the step of providing to said subject a second pharmaceutical composition comprising a conventional anti-HSV agent.

37. The method of claim 36, wherein said second pharmaceutical composition comprises acyclovir.

38. The method of claim 37, wherein said acyclovir is delivered via a route selected from the group consisting of topically, orally and intravenously.

39. A screening method for compounds having inhibitory activity against HSV U_S3 polypeptide-induced inhibition of apoptosis comprising the steps of:

(a) providing a first cell comprising an HSV U_S3 gene under the control of an HSV immediate early promoter;

(b) infecting said first cell with a herpes simplex virus that lacks a functional U_S3 gene;

(c) contacting said first cell with a test compound;

(d) incubating said first cell under conditions permitting viral replication; and

(e) comparing the cell pathology of said first cell following incubation with (i) the cell pathology of a second cell that lacks an HSV U_S3 gene following infection with said herpes simplex virus and (ii) the cell pathology of a third cell comprising an HSV U_S3 gene under the control of an HSV immediate early promoter following infection with said herpes simplex virus but in the absence of said test compound.

40. The method of claim 39, wherein said cell pathology comprises condensation of chromatin, obliteration of nuclear membranes, vacuolization, cytoplasmic blebbing and DNA fragmentation.

41. The method of claim 39, wherein said cell line contains an integrated copy of a wild-type HSV U_S3 gene under the control of an U_S3 promoter.

42. The method of claim 41, wherein said herpes simplex virus that lacks a functional HSV U_S3 gene has a deletion in both copies of the virally-encoded U_S3 genes.

43. The method of claim 42, wherein said herpes virus that lacks a functional HSV U_S3 gene carries a temperature sensitive mutation in both copies of the virally-encoded U_S3 gene and the incubation is at 39.5°C.

44. A screening method for compounds having inhibitory activity against HSV U_S3-induced inhibition of apoptosis comprising the steps of:

(a) providing a first cell comprising an HSV U_S3 gene under the control of an inducible promoter;

(b) inducing transcription from said promoter;

(c) contacting said first cell with a test compound;

(d) incubating said first cell under conditions permitting expression of an HSV U_S3 polypeptide; and

(e) comparing the cell pathology of said first cell following incubation with (i) the cell pathology of a second cell not having an HSV U_S3 gene following induction and (ii) the cell pathology of a third cell comprising an HSV U_S3 gene under the control of an inducible promoter following induction but in the absence of said test compound.

45. A screening method for compounds having inhibitory activity against HSV U_S3 polypeptide-induced inhibition of apoptosis comprising the steps of:

(a) providing a first cell;

(b) infecting said first cell with a herpes simplex virus;

(c) contacting said first cell with a test compound;

(d) incubating said first cell under conditions permitting viral replication; and

(e) comparing the cell pathology of said first cell following incubation with (i) the cell pathology of a second cell treated with said test compound alone and (ii) the cell pathology of a third cell following infection with said herpes simplex virus but in the absence of said test compound.

46. A method for expressing a polypeptide in a cell comprising the steps:

(a) contacting a cell with a herpes virus vector encoding said polypeptide;

(b) contacting said cell with an agent that blocks the transactivating function of U_S3 but does not block the apoptosis inhibiting function of U_S3.

47. A method for the identification of a candidate substance that is a modulator of US3-kinase activity comprising the steps of:

(a) providing an enzyme composition comprising U_S3 protein kinase and a threonine/serine protein substrate;

(b) contacting said composition with said candidate substance under conditions that allow phosphorylation;

(c) determining the phosphorylation of said threonine/serine substrate; and

(d) comparing the phosphorylation of said threonine/serine substrate with the phosphorylation of said threonine/serine substrate in the absence of a candidate inhibitor substance;

wherein an increase in phosphorylation is indicative of said candidate substance being a stimulator of phosphorylation and a decrease in phosphorylation of said threonine/serine substrate is indicative of said candidate being an inhibitor of phosphorylation.

48. An inhibitor of U_S3-kinase activity identified according to a method comprising the steps of:

(a) providing an enzyme composition comprising U_S3 protein kinase and a threonine/serine protein substrate;

(b) contacting said composition with said candidate substance under conditions that allow phosphorylation;

(c) determining the phosphorylation of said threonine/serine substrate; and

(d) comparing the phosphorylation of said threonine/serine substrate with the phosphorylation of said threonine/serine substrate in the absence of a candidate inhibitor substance;

wherein a decrease in phosphorylation of said threonine/serine substrate is indicative of said candidate being an inhibitor of phosphorylation.

49. A stimulator of U_S3-kinase activity identified according to a method comprising the steps of:

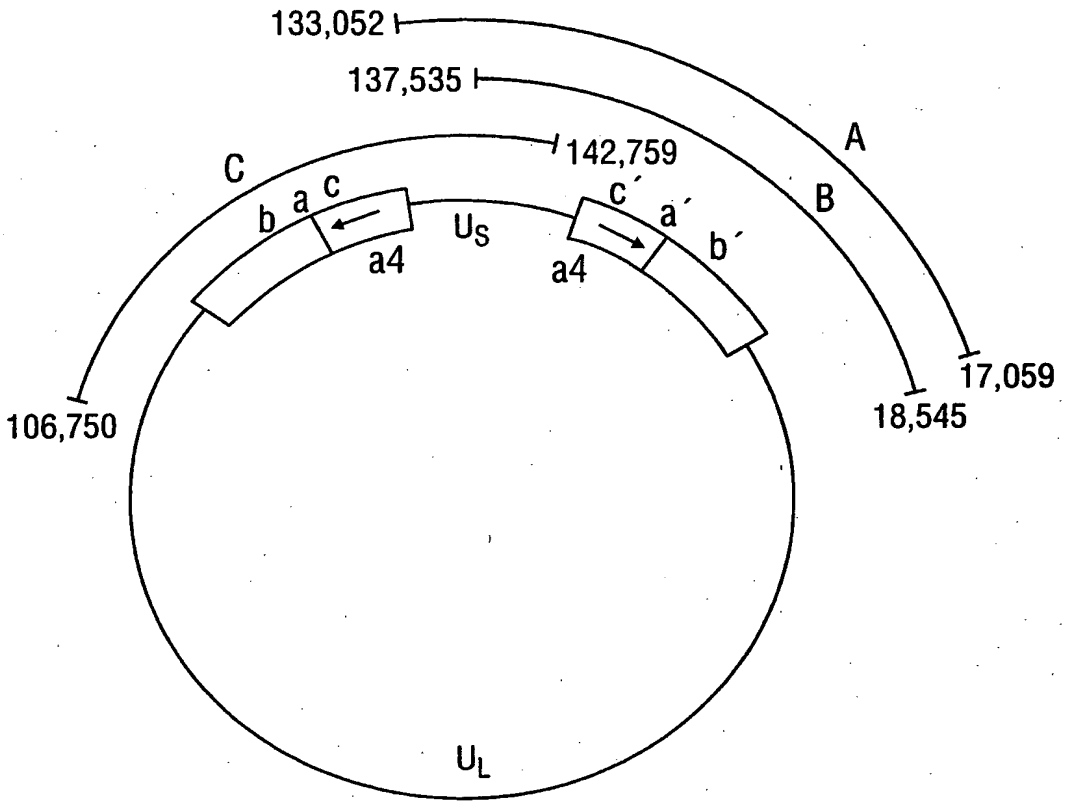
(a) providing an enzyme composition comprising U_S3 protein kinase and a threonine/serine protein substrate;

(b) contacting said composition with said candidate substance under conditions that allow phosphorylation;

(c) determining the phosphorylation of said threonine/serine substrate; and

(d) comparing the phosphorylation of said threonine/serine substrate with the phosphorylation of said threonine/serine substrate in the absence of a candidate inhibitor substance;

wherein an increase in phosphorylation is indicative of said candidate substance being a stimulator of phosphorylation.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B
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c
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Us
c´
a´
b´
a4
UL

FIG. 1

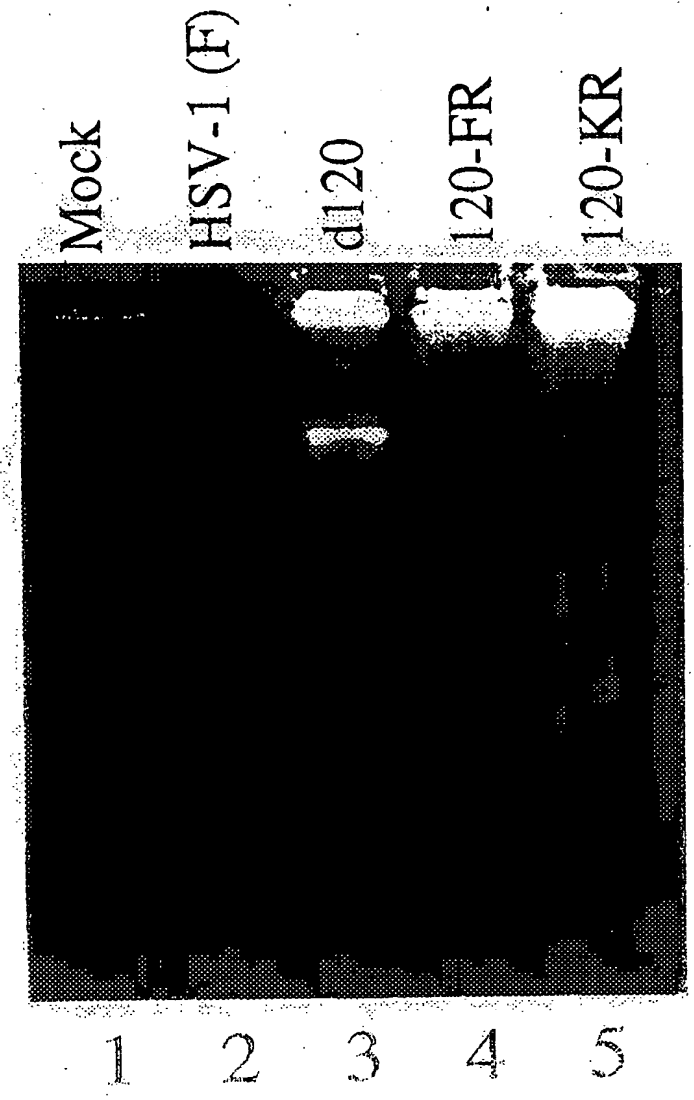
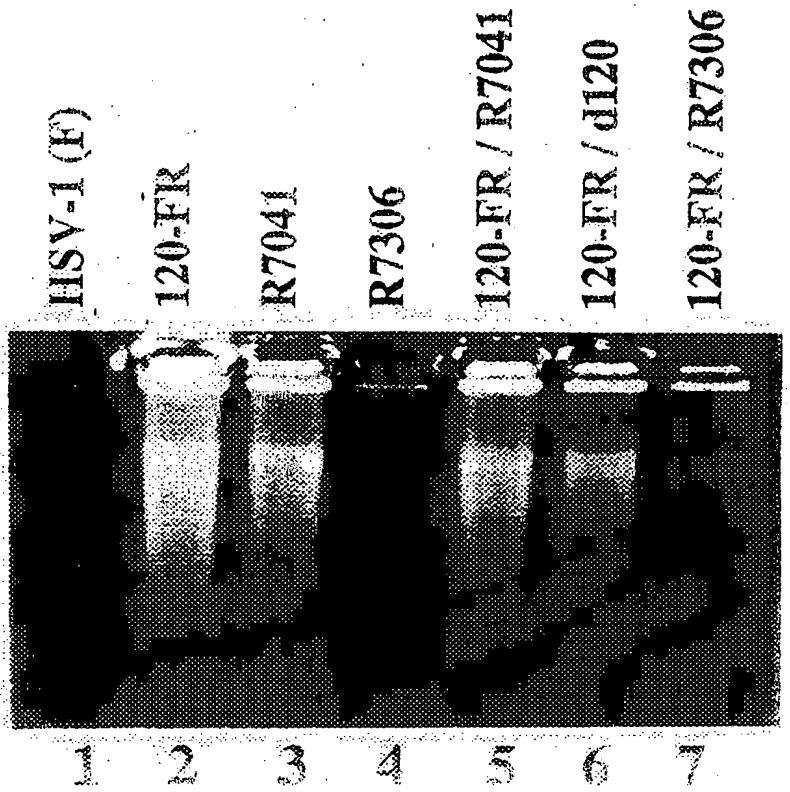


FIG. 2

FIG. 3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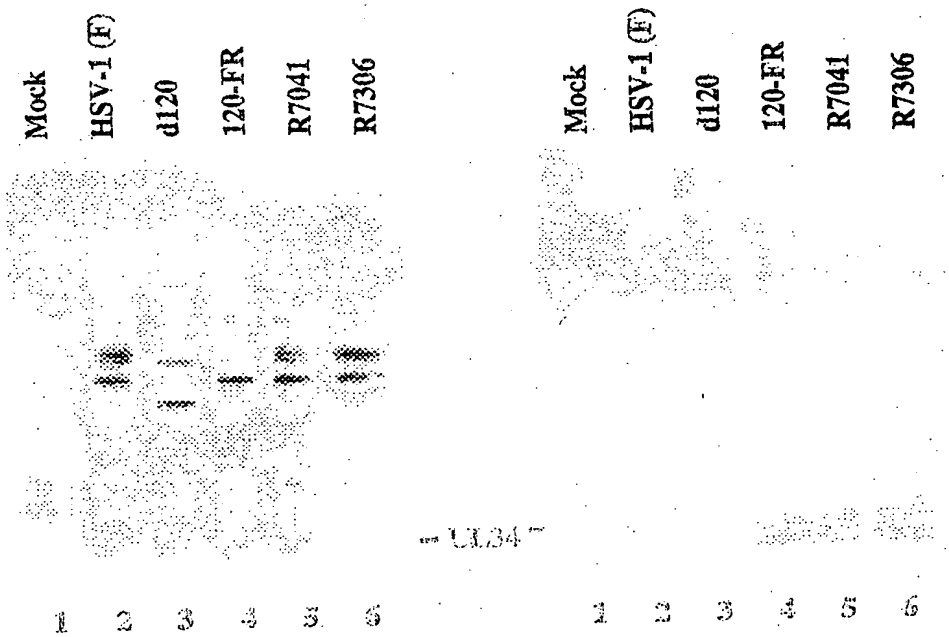
120-FR
R7041
R7306
120-FR / R7041
120-FR / d120
120-FR / R7306
1
2
3
4
5
6
7

FIG. 4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d120
120-FR
R7041
R7306
Mock
HSV-1 (F)
d120
120-FR
R7041
R7306
UL34
1 2 3 4 5 6
1 2 3 4 5 6

FIG. 5